

Best Available Copy

CTGTCTCAAGTCAGACAGCCTCATCTTACCTGTTCTTGCTACCCGTACCTTTGTGCGAT 1080
GATTCAGTTCTTTGAGCAGAGCTCTCTGCTGTGAACCCCTCAGCTGAATATCATCTGTAA 1140
ACGAAAAATCTGCACCGACAGGCACTCTGAGAGCTTCTCGAATGATCATGGCACAAGAA 1200
ATGTTCTCAGCTACTCTGACCCCTTTGTCATGCTTTGGGAAAGCTGTGAGTCTGTAAAAC 1260
CAGCCAGCACTCTGTTCTTCAGTCAACAACCTGCAGCTCTGCTTAATATGCTCTCTCAAG 1320
TACATCTTCAAGCTGCTGCTGAGCG 1344

LIGAND FOR THE c-KIT RECEPTOR AND METHODS OF USE THEREOF

5 This invention is a continuation-in-part application of U.S. Serial No. 549,306, filed October 5, 1990, which in turn is a continuation-in-part of U.S. Serial No. 573,483, filed August 27, 1990, the contents of which are hereby incorporated by reference into the present application.

10

The invention described herein was made in the course of work under Grant No. RO1-CA 32926 and ACS MV246D from the National Institute of Health and American Cancer Society, respectively. The United States Government has certain rights in this invention.

15

Background of the Invention

20 Throughout this application various publications are referred by arabic numerals to within parenthesis. Full bibliographic citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures for these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

25

30 The c-kit proto-oncogene encodes a transmembrane tyrosine kinase receptor for an unidentified ligand and is a member of the colony stimulating factor-1 (CSF-1) - platelet-derived growth factor (PDGF) - kit receptor subfamily (7, 41, 57, 23). c-kit was recently shown to be allelic with the white-spotting (W) locus of the mouse (9, 17, 35). Mutations at the W locus affect proliferation and/or migration and differentiation of germ cells, pigment cells and distinct cell populations of the hematopoietic system

35

-3-

a number of other W alleles are unable to proliferate in the fibroblast co-culture system in the absence of IL-3 (99, 100, 38). This suggested a function for the c-kit receptor in mature mast cells and implied that the ligand of the c-kit receptor is produced by fibroblasts. Huff and coworkers recently reported the stimulation of mast cell colonies from lymph node cells of mice infected with the nematode *Nippostrongylus brasiliensis* by using concentrated conditioned medium from NIH 3T3 fibroblasts (84). A short term mast cell proliferation assay was developed which means to purify a fibroblast derived activity (designated KL) which, in the absence of IL-3, supports the proliferation of normal BMMC's and peritoneal mast cells, but not W/W^V BMMC's. In addition, KL was shown to facilitate the formation of erythroid bursts (BFU-E). The biological properties of KL are in agreement with those expected of the c-kit ligand with regard to mast cell biology and aspects of erythropoiesis. The defect W mutations exert is cell autonomous; in agreement with this property, there is evidence for c-kit RNA expression in cellular targets of W mutations (35, 39). The recent characterization of the molecular lesions of several mutant alleles indicated that they are loss-of-function mutations that disrupt the normal activity or expression of the c-kit receptor (35, 100, 101, 36).

Mutations at the steel locus (Sl) on chromosome 10 of the mouse result in phenotypic characteristics that are very similar to those seen in mice carrying W mutations, i.e., they affect hematopoiesis, gametogenesis, and melanogenesis (5, 47, 51). Many alleles are known at the Sl locus; they are semidominant mutations, and the different alleles vary in their effects on the different cell lineages and their degree of severity (47, 51). The original Sl allele is a

-5-

bone marrow derived and connective tissue mast cells and in erythropoiesis, in combination with erythropoietin, KL promotes the formation of erythroid bursts (day 7-14 BFU-E). Furthermore, recent in vitro experiments with KL have demonstrated enhancement of the proliferation and differentiation of erythroid, myeloid and lymphoid progenitors when used in combination with erythropoietin, GM-CSF, GCSF and IL7 respectively suggesting that there is a role for the c-kit receptor system in progenitors of several hematopoietic cell lineages (27, 37).

Mutations at the steel locus on chromosome 10 of the mouse result in phenotypic characteristics that are very similar to those seen in mice carrying W mutations, i.e., they affect hematopoiesis, gametogenesis and melanogenesis (5, 47, 51). The ligand of the c-kit receptor, KL, was recently shown to be allelic with the murine steel locus based on the observation that KL sequences were found to be deleted in several severe S1 alleles (11, 38, 59). In agreement with the ligand receptor relationship between KL and c-kit, S1 mutations affect the same cellular targets as W mutations, however, in contrast to W mutations, S1 mutations are not cell autonomous and they affect the microenvironment of the c-kit receptor (12, 28, 30). Mutations at the steel locus are semidominant mutations and the different alleles vary in their effects on the different cell lineages and their degree of severity (47, 51). The original S1 allele is an example of a severe S1 mutation. S1/S1 homozygotes are deficient in germ cells, are devoid of coat pigment and they die perinatally of macrocytic anemia (5,50). Mice homozygous for the S1^d allele, although viable, have severe macrocytic anemia, lack coat pigment and are sterile (6). Both S1/+ and S1^d/+ heterozygotes have a diluted coat color and a moderate macrocytic anemia, but they are fertile,

-7-

Summary of Invention

5 This invention provides a nucleic acid molecule which encodes an amino acid sequence corresponding to a c-kit ligand (KL) and a purified c-kit ligand (KL) polypeptide.

10 A pharmaceutical composition which comprises the c-kit ligand (KL) purified by applicants or produced by applicants' recombinant methods and a pharmaceutically acceptable carrier is further provided as well as methods of treating patients which comprise administering to the patient the pharmaceutical composition of this invention.

-9-

fractions is shown below.

- A. Analysis of 0.5 ml fractions from analytical C18 column eluted with ammonium acetate buffer and 1-propanol gradient.
- B. Analysis of 0.5 ml fractions from analytical C4 column eluted with aqueous .1% TFA and absence of 2-mercapto-ethanol.

Figure 4.

Proliferation of W^{*} mutant mast cells in response to KL. Mast cells were derived from individual fetal livers from W/+ X W/+ mating, or bone marrow of wildtype, W^V and W⁴¹ heterozygotes and homozygotes. The proliferation characteristics of mutant mast cells was determined by using increasing concentrations of KL in a proliferation assay. Homozygous mutant mast cells are indicated by a solid line, heterozygotes mutant mast cells by a broken line and wildtype mast cells by a dotted line, except for W where normal fetuses may be either +/+ or W/+.

Figure 5.

Comparison of c-kit expression and growth factor responsiveness in BMNC and peritoneal mast cells (CTMC/PMC).

- A. Fluorescent staining of heparin proteoglycans in purified PMC and BMNC by using berberine sulfate.
- B. Determination of c-kit cell surface

-11-

- Figure 8. N-terminal amino acid sequence of KL and deduction of the corresponding nucleic acid sequence by PCR. Top line: N-terminal amino acid sequence (residues 10-36) of KL. Middle Line: Nucleotide sequences of three cDNAs obtained by cloning the 101 bp PCR product (see Figure 10) into M13 and subsequent sequence determination. Bottom Line: sequences of the degenerate sense and antisense primers used for first-strand cDNA synthesis and PCR. The amino acid sequence also is identified as SEQ ID:NO:2.
- Figure 9. Northern blot analysis using the PCR generated oligonucleotide probes corresponding to the isolated c-kit ligand polypeptide. A 6.5 kb mRNA was isolated with labelled probes.
- Figure 10. Derivation of cDNAs corresponding to the N-terminal amino acids 10-36 of KL by RT-PCR. One microgram of poly(A)⁺RNA from BALB/c 3T3 cells was used as template for cDNA synthesis and subsequent PCR amplification in combination with the two degenerate oligonucleotide primers. Electrophoretic analysis of the 101 bp PCR product in agarose is shown.
- Figure 11. Nucleotide Sequence and Predicted Amino Acid Sequence of the 1.4 kb KL cDNA clone. The predicted amino acid sequence of the long open reading frame is shown above and the nucleotide sequence using the single-letter

-13-

level of high c-kit protein.

- 5 B. Mast cells derived from bone marrow of +/+ or W/W^v adult mice or fetal liver cells of W/W or a normal littermate control (W/+ or +/+).

Figure 15. Coprecipitation and Cross-Linking of ¹²⁵I-KL with the c-kit receptor on mast cells.

- 10 A. Coprecipitation of KL with normal rabbit serum (NRS) or two anti-c-kit rabbit antisera (α-c-kit).

- 15 B. Cross-linking of KL to c-kit with disuccinimidyl substrate. SDS-page analysis was on either 12% or 7.5% polyacrylamide gels. Cross-linked species are labeled "KL + cK".

20 Figure 16. RFLP analysis of TaqI-digested DNA from S1/+ and SIISI mice. The S1 allele from C3HeB/Fej a/a CaJ S1 Hm mice was introduced into a C57BL/6J S1 Hm mice was introduced into a C57BL/6J background, and progeny of a C57BL/6J S1^{C3H} x S1^{C3H} cross were evaluated.

- 25 A. Hybridization of the 1.4 kB KL cDNA probe to DNA from two nonanemic (lanes SII+) and two anemic (lanes SIISI) mice. No hybridization to the DNA from the SIISI mice was detected.

- 30 B. Hybridization of the same blot to TIS Dra/SaI, a probe that is tightly linked to

-15-

using the primer combinations #1, + #2, #1 + #3 and #1 + #4. The reaction products were analyzed by electrophoresis in 1% NuSieve agarose gels in the presence of 0.25 μ g/ml ethidium bromide. The migration of ϕ X174 Hae III DNA markers is indicated.

Figure 19.

Topology of different KL protein products. Shaded areas delineate N-terminal signal peptides, solid black areas transmembrane domains and Y N-linked glycosylation sites. Dotted lines indicate the exon boundaries of the alternatively spliced exon and corresponding amino acid numbers are indicated. Arrows indicate the presumed proteolytic cleavage sites. The shaded region at the C-terminus of KL-S1^d indicates amino acids that are not encoded by KL. KL-S designates the soluble form of KL produced by proteolytic cleavage or the C-terminal truncation mutation of KL.

Figure 20.

Identification of KL-1 and KL-2 transcripts in different tissues by RNase protection assays. ³²P-labelled antisense riboprobe (625 nt.) was hybridized with 20 μ g total cell RNA from tissues and fibroblasts except for lung and heart where 10 μ g was used. Upon RNase digestion, reaction mixtures were analyzed by electrophoresis in a 4% polyacrylamide/urea gel. For KL-1 and KL-2 protected fragments of 575 nts. and 449 nts., are obtained respectively. Autoradiographic exposures were for 48 or 72

-17-

Figure 23. Panels A and B. Biosynthetic characteristics of KL-S1^d and KL-S protein products in COS cells.

5 Figure 24. Determination of biological activity in COS cell supernatants. Supernatants from COS cells transfected with the KL-1, KL-2, KL-S1^d and KL-S expression plasmids were assayed for activity in the mast cell proliferation assay. Serial dilutions of
10 supernatant were incubated with BMNCs and incorporation of ³H-thymidine was determined from 24-30 hours of culture.

15

-19-

relative of c-kit, shares the topological characteristics of KL and has been shown to be proteolytically cleaved to produce the soluble growth factor (44, 45). A recent analysis of the presumed structural characteristics of KL, furthermore indicates a relationship of KL and CSF-1 based on amino acid homology, secondary structure and exon arrangements indicating an evolutionary relationship of the two factors and thus strengthening the notion that the two receptor systems evolved from each other (4).

Alternatively spliced KL mRNAs which encode two different forms of the KL protein, i.e., KL-1 and KL-2, have recently been described (15). The KL encoded protein products have been defined and characterized in COS cells transfected with the KL cDNAs and extended the findings of Flanagan et al. in several ways. As noted hereinabove, KL is synthesized as a transmembrane protein which is proteolytically cleaved to produce the soluble form of KL. The protein product of the alternatively spliced transcript of KL, KL-2, which lacks the exon that encodes the presumptive proteolytic cleavage site was shown to display turnover characteristics that are distinct from those of KL-1. In addition, the proteolytic cleavage of both KL-1 and KL-2 can be regulated by agents such as PMA and the calcium ionophore A23187. The relative abundance of KL-1 and KL-2 has been determined in a wide variety of different mouse tissues. This indicates that the expression of KL-1 and KL-2 is controlled in a tissue specific manner.

The gene products of the Sl^d allele have also been defined (15). Sl^d results from a deletion within KL which includes the sequences encoding the transmembrane and cytoplasmic domains of the protein resulting in a biologically active, secreted mutant KL protein. The respective roles of the

-21-

known to those of skill in the art.

Also provided by this invention is a pharmaceutical composition which comprises an effective amount of the purified mammalian protein corresponding to c-kit ligand described hereinabove and a pharmaceutically acceptable carrier.

Further provided is a pharmaceutical composition for the treatment of leucopenia in a mammal comprising an effective amount of the above mentioned pharmaceutical composition and an effective amount of a hemopoietic factor, wherein the factor is selected from the group consisting of GCSF, GMCSF and IL-3, effective to treat leucopenia in a mammal.

Also provided by this invention is a pharmaceutical composition for the treatment of anemia in a mammal, which comprises an effective amount of the pharmaceutical composition described hereinabove and an effective amount of EPO (erythropoietin) or IL-3, effective to treat anemia in a mammal. Anemia encompasses, but is not limited to Diamond Black fan anemia and aplastic anemia. However, for the treatment of Black fan anemia and aplastic anemia, a pharmaceutical composition comprising an effective amount of the composition described hereinabove and an effective amount of G-CSF and GM-CSF, effective to treat anemia is preferred. A method of treating anemia in mammals by administering to the mammals the above composition is further provided by this invention. A pharmaceutical composition effective for enhancing bone marrow during transplantation in a mammal which comprises an effective amount of the pharmaceutical composition described hereinabove, and an effective amount of IL-1 or IL-6, effective to enhance engraftment of bone marrow during

-23-

effective amount of the purified mammalian protein corresponding to c-kit ligand and a pharmaceutically acceptable carrier, effective to inhibit the loss of pigment in the subject's hair.

5

Methods of treating the above-listed disorders by the administration of the effective composition, in an amount effective to treat that disorder, also is provided.

10

As used herein, the terms "subject" shall mean, but is not limited to, a mammal, animal, human, mouse or a rat. "Mammal" shall mean, but is not limited to meaning a mouse (murine) or human.

15

This invention provides an isolated nucleic acid molecule which encodes an amino acid sequence corresponding to a c-kit ligand (KL). Examples of such nucleic acids include, but are not limited to the nucleic acids designated KL 1.4, KL-1, KL-2 or S-KL. The invention also encompasses nucleic acids molecules which differ from that of the nucleic acid molecule which encode these amino acid sequences, but which produce the same phenotypic effect. These altered, but phenotypically equivalent nucleic acid molecules are referred to as "equivalent nucleic acids". And this invention also encompasses nucleic acid molecules characterized by changes in non-coding regions that do not alter the phenotype of the polypeptide produced therefrom when compared to the nucleic acid molecule described hereinabove. This invention further encompasses nucleic acid molecules which hybridize to the nucleic acid molecule of the subject invention. As used herein, the term "nucleic acid" encompasses RNA as well as single and double-stranded DNA and cDNA. In addition, as used herein, the term "polypeptide" encompasses any naturally occurring allelic

20

25

30

-25-

To recover the protein when expressed in *E. coli*, *E. coli* cells are transfected with the claimed nucleic acids to express the c-kit ligand protein. The *E. coli* are grown in one (1) liter cultures in two different media, LB or TB and pelleted. Each bacterial pellet is homogenized using two passages through a French pressure cell at 20'000 lb/in² in 20 ml of breaking buffer (below). After a high speed spin (120k rpm x 20 minutes) the supernatants were transferred into a second tube. The c-kit protein or polypeptide is located in the particulate fraction. This may be solubilized using 6M guanidium-HCl or with 8M urea followed by dialysis or dilution.

Breaking Buffer

50 mM Hepes, pH 8.0
20% glycerol
150 mM NaCl
1 mM Mg So₄
2 mM DTT
5mM EGTA
20 µg/ml DNase I.

A purified soluble c-kit ligand (KL) polypeptide as well as a fragment of the purified soluble c-kit ligand (KL) polypeptide is further provided by this invention.

In one embodiment of this invention, the c-kit ligand polypeptide corresponds to amino acids 1 to 164. In other embodiments of this invention, the c-kit ligand polypeptide corresponds to amino acids 1 to about 148, or fusion polypeptides corresponding to amino acids 1 to about 148 fused to amino acids from about 165 to about 202 or 205, as well as a fusion polypeptide corresponding to amino acids 1

-27-

Alternatively, the conjugated polypeptide may be administered to a patient, for example, by intravenous administration. A sufficient amount of the conjugated polypeptide must be administered, and generally such amounts will vary depending upon the size, weight, and other characteristics of the patient. Persons skilled in the art will readily be able to determine such amounts.

Subsequent to administration, the conjugated polypeptide which is bound to any c-kit receptor present on the surface of cells or tissue is detected by intracellular imaging.

In the method of this invention, the intracellular imaging may comprise any of the numerous methods of imaging, thus, the imaging may comprise detecting and visualizing radiation emitted by a radioactive isotope. For example, if the isotope is a radioactive isotope of iodine, e.g., ^{125}I , the detecting and visualizing of radiation may be effected using a gamma camera to detect gamma radiation emitted by the radioiodine.

In addition, the soluble, c-kit ligand (KL) polypeptide fragment may be conjugated to a therapeutic agent such as toxins, chemotherapeutic agents or radioisotopes. Thus, when administered to a patient in an effective amount, the conjugated molecule acts as a tissue specific delivery system to deliver the therapeutic agent to the cell expressing c-kit receptor.

A method for producing a c-kit ligand (KL) polypeptide is also provided which comprises growing the host vector system described hereinabove under suitable conditions permitting production of the c-kit ligand (KL) polypeptide and recovering the resulting c-kit ligand (KL) polypeptide.

-29-

A method of modifying a biological function associated with c-kit cellular activity is provided by this invention. This method comprises contacting a sample of the cell, whose function is to be modified, with an effective amount of a pharmaceutical composition described hereinabove, effective to modify the biological function of the cell. Biological functions which may be modified by the practice of this method include, but are not limited to cell-cell interaction, propagation of a cell that expresses c-kit and in vitro fertilization. This method may be practiced in vitro or in vivo. When the method is practiced in vivo, an effective amount of the pharmaceutical composition described hereinabove is administered to a patient in an effective amount, effective to modify the biological function associated with c-kit function.

This invention also provides a method of stimulating the proliferation of mast cells in a patient which comprises administering to the patient the pharmaceutical composition described hereinabove in an amount which is effective to stimulate the proliferation of the mast cells in the patient. Methods of administration are well known to those of ordinary skill in the art and include, but are not limited to administration orally, intravenously or parenterally. Administration of the composition will be in such a dosage such that the proliferation of mast cells is stimulated. Administration may be effected continuously or intermittently such that the amount of the composition in the patient is effective to stimulate the proliferation of mast cells.

A method of inducing differentiation of mast cells or erythroid progenitors in a patient which comprises administering to the patient the pharmaceutical composition

-31-

melanoma. Methods of administration are well known to those of ordinary skill in the art and include, but are not limited to administration orally, intravenously or parenterally. Administration of the composition will be in such a dosage such that melanoma is treated. Administration may be effected continuously or intermittently such that the amount of the composition in the patient is effective.

The soluble, c-kit ligand (KL) polypeptide may also be mutated such that the biological activity of c-kit is destroyed while retaining its ability to bind to c-kit. Thus, this invention provides a method of treating allergies in a patient which comprises administering to the patient an effective amount of the soluble, mutated c-kit ligand described hereinabove and a pharmaceutically acceptable carrier, in an amount which effective to treat the allergy. As is well known to those of ordinary skill in the art, the amount of the composition which is effective to treat the allergy will vary with each patient that is treated and with the allergy being treated. Administration may be effected continuously or intermittently such that the amount of the composition in the patient is effective.

Furthermore, this invention provides a method for measuring the biological activity of a c-kit (KL) polypeptide which comprises incubating normal bone-marrow mast cells with a sample of the c-kit (KL) polypeptide which comprises incubating normal bone-marrow mast cells with sample of the c-kit ligand (KL) polypeptide under suitable conditions such that the proliferation of the normal bone-marrow mast cells are induced; incubating doubly mutant bone-marrow mast cells with a sample of the c-kit ligand (KL) polypeptide under suitable conditions; incubating each of the products thereof with ³H-thymidine; determining the amount of thymidine

-33-

Mast cell cultures, preparation of peritoneal mast cell and flow cytometry

5 Mast cells were grown from bone marrow of adult mice and fetal liver cells of day 14-15 fetuses in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), conditioned medium from WEHI-3B cells, non-essential amino acids, sodium pyruvate, and 2-mercapto-ethanol (RPMI-Complete (C)) (60). Non-adherent cells were harvested, refed weekly and
10 maintained at a cell density less than 7×10^5 cells/ml. Mast cell content of cultures was determined weekly by staining cytospin preparations with 1% toluidine blue in methanol. After 4 weeks, cultures routinely contained greater than 95% mast cells and were used from proliferation
15 assays. Peritoneal mast cells were obtained from C57B1/6 mice by lavage of the peritoneal cavity with 7-10 ml of RPMI-C. Mast cells were purified by density gradient centrifugation on 22% Metrizamide (Nycomed, Oslo, Norway) in PBS without Ca^{++} and Mg^{++} , essentially as previously
20 described (61). Mast cells were stained with 1% toluidine blue in methanol for 5 minutes and washed for 5 minutes in H_2O , and berberine sulfate by standard procedures (62). Mast cells were labeled with c-kit specific rabbit antisera which recognizes extracellular determinants of c-kit as
25 previously described and analyzed on a FACSCAN (Becton Dickinson) (38).

Mast cell proliferation assay

30 Mast cells were washed three times in RPMI to remove IL-3 and cultured at a concentration of 5×10^4 c/ml in RPMI-C in a volume of .2 ml in 96 well plates with two fold serial dilutions of test samples. Plates were incubated for 24 hours at 37°C , 2.5 μC of ^3H -TdR was added per well and

-35-

equilibrated with PBS and calibrated with molecular weight markers; bovine serum albumin (Mr 68,000), chymotrypsinogen (Mr 25,700), and ribonuclease A (Mr 14,300), all obtained from Pharmacia, Piscataway, NJ. The concentrate from the Blue Agarose column was loaded onto the gel filtration column, the flow rate adjusted to 37.5 ml/hour and 7.5 ml fractions collected.

Anion exchange and reverse-phase HPLC (RP-HPLC)

High performance liquid chromatography was performed using a Waters HPLC system (W600E Powerline controller, 490E programmable multiwavelength detector, and 810 Baseline Workstation, Waters, Bedford, MA). Active fractions from gel filtration were dialyzed in 0.05 M Tris-HCl pH 7.8 and loaded onto a Protein-Pak™ DEAE-5PW HPLC column (7.5 mm X 7.5 cm, Waters), equilibrated with 0.05 M Tris-HCl pH 7.8. Bound proteins were eluted with a linear gradient from 0 to 0.05 M Tris-HCl pH 7.8. Bound proteins were eluted with a linear gradient from 0 to 0.4M NaCl in .02 M Tris-HCl pH 7.8. The flow rate was 1 ml/minute and 2 ml fractions were collected.

RP-HPLC was performed using a semi-preparative and an analytical size C₁₈ column from Vydac. For both columns buffer A was 100 mM ammonium acetate pH 6.0, and buffer B was 1-propanol. The biologically active fractions from anion exchange were pooled and loaded onto the semi-preparative C₁₈ column. Bound proteins were eluted with a steep gradient of 0% - 23% 1-propanol within the first 10 minutes and 23-33% 1-propanol in 70 minutes. The flow rate was adjusted to 2 ml/min and 2 ml fractions were collected. Biologically active fractions were pooled and diluted 1:1 with buffer A and loaded on the analytical C₁₈ reverse phase

-37-

Experimental MethodsShort term mast cell proliferation assay detects a fibroblast derived activity

5 In order to identify and measure a fibroblast derived growth factor activity which facilitates the proliferation of normal but not $\underline{W}/\underline{W}^V$ mast cells, BMMC were washed free of IL-3 containing medium, incubated with medium containing 20 fold concentrated fibroblast conditioned medium (FCM) or WEHI-3 CM (IL-3) and after 24 hours of incubation ^3H -thymidine incorporation was determined. The response of BMMC derived from normal $+/+$ and mutant $\underline{W}/\underline{W}^V$ mice to IL-3 was similar (Figure 1); in contrast, 20 fold concentrated fibroblast conditioned medium facilitated the proliferation of $+/+$ mast cells, but little proliferation was seen with $\underline{W}/\underline{W}^V$ mast cells. Concentrated FCM was also tested for its ability to stimulate the proliferation of other IL-3 dependent cells. The myeloid 32D cells are known to lack c-kit gene products (35). No proliferation of the 32D cells was observed with FCM, although normal proliferation was obtained with WEHI-3 CM (not shown). Taken together these results and the known defects in c-kit for both the \underline{W} and \underline{W}^V alleles (38), suggested that FCM activity was dependent on the expression of a functional c-kit protein in mast cells (BMMC) and therefore might be the ligand of the c-kit receptor. In addition the FCM activity was distinct from IL-3. Therefore, normal and \underline{W} mutant mast cells provide a simple, specific assay system for the purification of the putative c-kit ligand (KL) from fibroblast conditioned medium.

-39-

correlated well with the peak of biological activity (Figure 3). There was no significant difference in the migration of this band under reduced and non-reduced conditions, indicating that KL was not a disulfide linked dimer (Figure 3C). Three discrete species were observed on both reduced and non-reduced SDS-PAGE indicating size heterogeneity of the purified material. The total amount of protein estimated by absorbance at 280 nm correlated with the amount detected by silver stain relative to BSA as a reference standard. As indicated in Table 1, the purification of KL from conditioned medium of Balb/3T3 cells was more than 3000 fold and the recovery of the initial total activity 47%. Half maximal proliferation of +/- mast cells in applicants' assay volume of 0.2 ml is defined as 50 units of activity and corresponds to approximately 0.5 ng of protein. Isoelectric focusing of partially purified material (after ion exchange) revealed a major peak of activity in the pH range of 3.7-3.9 indicating an isoelectric point for KL of 3.7-3.9.

20

-41-

display a minor response in the co-culture assay, and homozygotes for the less severe \underline{W}^{41} allele have a moderate anemia and their mast cells show an intermediate response in the co-culture assay. Homozygous and heterozygous mutant and +/+ mast cells were derived from the bone marrow for the \underline{W}^V and \underline{W}^{41} alleles and from day 14 fetal livers for the \underline{W} allele as described previously (38). Fetal liver derived $\underline{W}/\underline{W}$ mast cells did not proliferate in response to KL whereas both heterozygous ($\underline{W}/+$) and normal (+/+) mast cells displayed a similar proliferative response to KL (Figure 4). Bone marrow derived mast cells from $\underline{W}^V/\underline{W}^V$ mice were severely defective in their response to KL, although some proliferation, 10% of +/+ values, was observed at 100 U/ml (Figure 4). $\underline{W}^V/+$ mast cells in contrast to heterozygous $\underline{W}/+$ mast cells showed an intermediate response (40%) in agreement with the dominant characteristics of this mutation. $\underline{W}^{41}/\underline{W}^{41}$ and $\underline{W}^{41}/+$ mast cells were also defective in their ability to proliferate with KL, although less pronounced than mast carrying the \underline{W} and the \underline{W}^V alleles, which is consistent with the in vivo phenotype of this mutation (Figure 4). These results indicate a correlation of the responsiveness of mast carrying the \underline{W} , \underline{W}^V and \underline{W}^{41} alleles to KL with the severity and in vivo characteristics of these mutations. In contrast, the proliferative response of mutant mast cells to WEHI-3CM (IL-3) was not affected by the different \underline{W} mutations.

KL stimulates the proliferation of peritoneal mast cells

Mast cells of the peritoneal cavity (PMC) have been well characterized and in contrast to BMNC represent connective tissue-type mast cells (66). PMC do not proliferate in response to IL-3 alone; however, their mature phenotype and viability can be maintained by co-culture with NIH/3T3

-43-

KL stimulates the formation of erythroid bursts (BFU-E)

5 An important aspect of W mutations is their effect on the erythroid cell lineage. The in vivo consequences of this defect are macrocytic anemia which is lethal for homozygotes of the most severe alleles (47, 65). Analysis of erythroid progenitor populations in the bone marrow of W/W^V mice indicates a slight decrease of BFU-E and CFU-E (68,69). In
10 livers of W/W fetuses the number of BFU-E is not affected but a large decrease in the number of CFU-E is seen suggesting a role for c-kit at distinct stages of erythroid maturation presumably prior to the CFU-E stage (35). In order to evaluate a role for KL in erythropoiesis and to further define its relationship to the c-kit receptor, the
15 effect of KL on BFU-E formation was determined. Bone marrow, spleen and fetal liver cells were plated, by using standard culture conditions, in the presence and absence of KL, erythropoietin and WEHI-3 CM. BFU-E were then scored on day 7 of culture. In the absence of erythropoietin, no
20 erythroid growth was observed with either WEHI-3 CM or KL. In the presence of erythropoietin, BFU-E from spleen cells were stimulated by KL in a dose dependent manner, from 12 BFU-E/10⁶ cells with erythropoietin alone to 50 BFU-E/10⁶ cells with maximal stimulation at 2.5 ng of KL/ml (Figure
25 6). In addition to the effect on the number of BFU-E, the average size of the bursts was dramatically increased by KL. The number of BFU-E obtained by using spleen cells with KL + erythropoietin was similar to the number observed with WEHI-3 CM + erythropoietin. In contrast, KL +
30 erythropoietin did not stimulate the proliferation of BFU-E from bone marrow cells, whereas WEHI-3 CM + erythropoietin induced the formation of 18 BFU-E from 10⁵ bone marrow cells. The effect of KL on day 14 fetal liver cells was also examined and similar results were observed as with

-45-

of KL to W mutant mast cells correlates with c-kit expression on the cell surface, V, 37(+) versus W(-).

Determination of the peptide sequence of the c-kit ligand

5

The c-kit receptor protein was isolated as described hereinabove and the sequence of the protein was determined by methods well known to those of ordinary skill in the art.

-47-

ability to stimulate the proliferation of BMNC and purified peritoneal mast cells (CTMC), but not BMNCs from W mutant mice. Balb/3T3 fibroblasts are a source for the hematopoietic growth factors G-CSF, GM-CSF, CSF-1, LIF and IL-6; however, none of these have the biological activities of KL (35, 71). Furthermore, preliminary results from the determination of the protein sequence of KL indicate that KL is different from the known protein sequences.

An essential role for c-kit and its ligand in the proliferation, differentiation, and/or survival of mast cells in vivo has been inferred because of the absence of mast cells in W mutant mice (72, 73). The precise stage(s) at which c-kit function is required in mast cell differentiation are not known. Mast cells derived in vitro from bone marrow, fetal liver, or spleen with IL-3 resemble mucosal mast cells (MMC), although they may represent a precursor of both types of terminally differentiated mast cells, MMC and CTMC (66). Apparently, c-kit is not required for the generation of BMNC from hematopoietic precursors since IL-3 dependent mast cells can be generated with comparable efficiency from bone marrow or fetal liver of both normal and W mutant mice (60). The demonstration of c-kit expression in BMNC and CTMC/PMC and the corresponding responsiveness of BMNC and mature CTMC/PMC to KL suggests a role for c-kit at multiple stages in mast cell differentiation. In addition to fibroblasts, it has been shown that the combination of IL-3 and IL-4, IL-3 and PMA, or crosslinking of IgE receptors can stimulate the proliferation of CTMC in vitro (74, 75, 76, 77, 78). In contrast to these biological response modifiers, which are mediators of allergic and inflammatory responses, KL by itself in the presence of FCS is capable of stimulating CTMC proliferation. Therefore, KL may have a mast cell

-49-

stimulate an earlier erythroid-multipotential precursor in bone marrow which appears at later times in culture (day 14-20). To demonstrate a direct effect of KL on BFU-E formation and to rule out the involvement of accessory cells or other endogenous growth factors, experiments with purified progenitor populations need to be performed.

In addition to the defects in erythropoiesis and mast cell development, W mutations are thought to affect the stem cell compartment of the hematopoietic system. The affected populations may include the spleen colony forming units (CFU-S) which produce myeloid colonies in the spleen of lethally irradiated mice as well as cell with long term repopulation potential for the various cell lineages (81, 46, 47, 81, 82). It will now be of interest to determine if there is an effect of KL in the self-renewal or the differentiation potential of hematopoietic stem cell populations, possibly in combination with other hematopoietic growth factors, in order to identify the stage(s) where the c-kit/W gene product functions in the stem cell compartment.

Mutations at the steel locus (S1) of the mouse produce pleiotropic phenotypes in hematopoiesis, melanogenesis and gametogenesis similar to those of mice carrying W mutations (47, 51). However, in contrast to W mutations, S1 mutations affect the microenvironment of the cellular target of the mutation and are not cell autonomous (46). Because of the parallel and complementary effects of the W and the S1 mutations, it has been suggested that the S1 gene encode the ligand of the c-kit receptor or a gene product that is intimately linked to the production and/or function of this ligand (9). In agreement with this conjecture S1/S1^d embryo fibroblasts or conditioned medium from S1/S1^d fibroblasts

-51-

Complete) (36,60). BALB/c 3T3 cells (1) were obtained from Paul O'Donnell (Sloan-Kettering Institute, New York, New York) and were grown in Dulbecco's modified MEM supplemented with 10% calf serum, penicillin, and streptomycin.

5

Purification and amino acid sequence determination of KL

KL was purified from conditioned medium of BALB/c 3T3 cells by using a mast cell proliferation assay as described elsewhere (37). Conditioned medium was then concentrated 100- to 200-fold with a Pellicon ultrafiltration apparatus followed by an Amicon stirred cell. The concentrate was then chromatographed on Blue Agarose (Bethesda Research Laboratories, Gaithersburg, MD), and the flow-through, which contained the active material, was concentrated in dialysis tubing with polyethylene glycol 8000 and then fractionated by gel filtration chromatography on an ACA54 Ultrogel (LKB, Rockland, MD) column. The biological activity eluted as a major and a minor peak, corresponding to 55-70 kd and 30 kd, respectively. The fractions of the main peak were pooled, dialyzed, and fractionated by FPLC on a DEAE-5PW column with an NaCl gradient. The activity eluted at 0.11 M NaCl from the FPLC column. Peak fractions were pooled and subjected to HPLC with a semi-preparative C18 column and an ammonium acetate-n-propanol gradient. The active material eluted at 30% n-propanol from the semipreparative C18 column was diluted 1:1 and re-chromatographed by using an analytical C18 column. A single peak of activity eluted again at 30% n-propanol, which corresponded to a major peak of absorbance (280nm) in the eluant profile. Similar results were obtained by using a C4 column with H₂O and acetonitrile containing 0.1% TFA as solvents. N-terminal amino acid sequence was determined on an Applied Biosystems 477A on-line PTH amino acid analyzer (Hewick et al., 1961).

-53-

20 mM Tris (pH 7.4), 150 mM NaCl, 20 mM EDTA, 10% glycerol, and protease inhibitors phenylmethylsulfonyl fluoride (1mM) and leupeptin (20 μ g/ml). Lysates were immunoprecipitated with normal rabbit serum, or c-kit specific sera raised by immunization of rabbits with a fragment of the v-kit tyrosine kinase domain (23); or the murine c-kit expressed from a cDNA in a recombinant vaccinia virus (36). For coprecipitation experiments, immunoprecipitates were washed three times with wash A (0.1% Triton X-100, 20 mM Tris [pH 7.4], 150 mM NaCl, 10% glycerol), solubilized in SDS sample buffer, and analyzed by SDS-PAGE and autoradiography. For cross-linking experiments, cells were incubated with disuccinimidyl substrate (0.25 mg/ml) in PBS for 30 minutes at 4°C, washed in PBS, and lysed as described above. Washing conditions following precipitation were as follows: one time in wash B (50 mM Tris, 500 mM NaCl, 5 mM EDTA, 0.2% Triton X-100), three times in wash C (50 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 5mM EDTA), and one time in wash D (10 mM Tris, 0.1% Triton X-100).

cDNA synthesis, PCR amplification (RT-PCR), and sequence determination

The RT-PCR amplification was carried out essentially as described (53). For cDNA synthesis, 1 μ g of poly(A)⁺ RNA from confluent BALB/c 3T3 cells in 25 μ l of 0.05 M Tris-HCl (pH 8.3), 0.075 M KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 200 μ M dNTPs and 25 U of RNasin (Promega) was incubated with 50 pmol of antisense primer and 50 U of Moloney murine leukemia virus reverse transcriptase at 40°C for 30 minutes. Another 50 U of reverse transcriptase was added, and incubation was continued for another 30 minutes. The cDNA was amplified by bringing up the reaction volume to 50 μ l with 25 μ l of 50 mM KCl, 10mM Tris-HCl(pH 8.3), 1.5 mM MgCl₂, 0.01% (w/v)

-55-

Chi-gwin et al. (10). Total cellular RNA (10 μ g) and poly(A)⁺ RNA were fractionated in 1% agarose-formaldehyde gels and transferred to nylon membranes (Nytran, Schleicher & Schuell); prehybridization and hybridization were performed as previously described (86, 35). The 1.4 kb KL cDNA labeled with [³²P]phosphate was used as a probe for hybridization (87).

Preparation of c-kit and c-kit ligand monoclonal antibodies

For the isolation of human monoclonal antibodies, eight week old Balb/c mice are injected intraperitoneally with 50 micrograms of a purified human soluble c-kit ligand (KL) polypeptide, or a soluble fragment thereof, of the present invention (prepared as described above) in complete Freund's adjuvant, 1:1 by volume. Mice are then boosted, at monthly intervals, with the soluble ligand polypeptide or soluble ligand polypeptide fragment, mixed with incomplete Freund's adjuvant, and bled through the tail vein. On days 4, 3, and 2 prior to fusion, mice are boosted intravenously with 50 micrograms of polypeptide or fragment in saline. Splenocytes are then fused with non-secreting myeloma cells according to procedures which have been described and are known in the art to which this invention pertains. Two weeks later, hybridoma supernatants are screened for binding activity against c-kit receptor protein as described hereinabove. Positive clones are then isolated and propagated.

Alternatively, to produce the monoclonal antibodies against the c-kit receptor, the above method is followed except that the method is followed with the injection and boosting of the mice with c-kit receptor protein.

-57-

the correct amino acid sequence (Figure 8). An oligonucleotide (49 nucleotides) corresponding to the unique sequence of the PCR products was then used to screen a λ gt11 mouse fibroblast library. A 1.4 kb clone was obtained that, in its 3' half, specifies an open reading frame that extends to the 3' end of the clone and encodes 270 amino acids (Figure 11). The first 25 amino acids of the KL amino acid sequence have the characteristics of a signal sequence. The N-terminal peptide sequence that had been derived from the purified protein (amino acids 26-65) follows the signal sequence. A hydrophobic sequence of 21 amino acids (residues 217-237) followed at its carboxyl end by positively charged amino acids has the features of a transmembrane segment. In the sequence between the signal peptide and the transmembrane domain, four potential N-linked glycosylation sites and four irregularly spaced cysteines are found. A C-terminal segment of 33 amino acids follows the transmembrane segment without reaching a termination signal (end of clone). The KL amino acid sequence therefore has the features of a transmembrane protein: an N-terminal signal peptide, an extracellular domain, a transmembrane domain, and a C-terminal intracellular segment.

RNA blot analysis was performed to identify KL-specific RNA transcripts in BALB/c 3T3 cells (Figure 12). A major transcript of 6.5 kb and two minor transcripts of 4.6 and 3.5 kb were identified on a blot containing poly(A)⁺ RNA by using the 1.4 kb KL cDNA as a probe. Identical transcripts were detected by using an end-labeled oligonucleotide derived from the N-terminal protein sequence. This result then indicates that KL is encoded by a large mRNA that is abundantly expressed in BALB/c 3T3 cells.

-59-

c-kit receptor, it was determined if receptor-ligand complexes could be purified by immunoprecipitation with c-kit antisera. This experiment requires that a KL-c-kit complex be stable and not be affected by the detergents used for the solubilization of the c-kit receptor. Precedent for such properties of receptor-ligand complexes derives from the closely related macrophage colony-stimulating factor (CSF-1) receptor and PDGF receptor systems (89). ^{125}I -KL was bound to receptors on BMBC by incubation at 4°C. Upon washing to remove free ^{125}I -KL, the cells were solubilized by using the Triton X-100 lysis procedure and precipitated with anti-v-kit and anti-c-kit rabbit sera conjugated to protein A-Sepharose. ^{125}I -KL was retained in immunoprecipitates obtained by incubation with anti-kit sera but not with nonimmune controls, as shown by the analysis of the immune complexes by SDS-PAGE (Figure 15A), where recovery of intact ^{125}I -KL was demonstrated from the samples containing the immune complexes prepared with anti-kit sera.

To further characterize the c-kit-KL receptor-ligand complexes, it was determined whether KL could be cross-linked to c-kit. BMBC were incubated with ^{125}I -KL, washed and treated with the cross-linked disucciminidyl substrate. Cell lysates were then immunoprecipitated with anti-v-kit antiserum and analyzed by SDS-PAGE. Autoradiography indicated three species: one at approximately 30 kd, representing KL coprecipitated by not cross-linked to c-kit; one at 180-190 kd, corresponding to a covalently linked c-kit-KL monomer-monomer complex; and a high molecular weight structure that is at the interface between the separating and stacking gels (Figure 15B). Molecular structures of similar size were observed if the cell lysates were separated directly on SDS-PAGE without prior immunoprecipitation. Following precipitation with nonimmune

-61-

insertion site and S1, this result is consistent with the notion that KL maps to the S1 locus.

Table 2. Mapping of the Position of the KL Gene by Linkage Analysis Using an Interspecific Cross

Probe	Progeny			
	Nonrecombinant		Recombinant	
1.4 kb KL cDNA	B6	Sp	B6	Sp
TIS Dra/SaI	B6	Sp	Sp	B6
	32	20	0	1
n = 53	% recombination = 1.9 ± 1.9			

The concordance of inheritance of C57Bl/6J (B6) or M. spretus (Sp) alleles in progeny of an interspecific cross (see Experimental Procedures) was determined by scoring for TaqI RFLPs of the KL 1.4 kb cDNA probe and TIS Dra/SaI (a probed from a transgene insertion site that is tightly linked to S1; see Results). Percent recombination was calculated according to Green (1981).

The locus identified by KL was also examined in mice that carry the original S1 mutation (50). For this purpose, the observation that the transgene insertion site locus is polymorphic in inbred strains was taken advantage of, and was utilized to determine the genotype at S1 during fetal development. C57BL/6J mice that carry the S1 mutation maintained in the C3HeB/FeJ strain were generated by mating, and F1 progeny carrying the S1 allele were intercrossed (C57BL/6J S1^{3CH/+} S1^{3CH/+}). Homozygous SIISI progeny from

-63-

and the c-kit protein. KL-specific cDNA clones were derived and it was shown that KL maps to the S1 locus on mouse chromosome 10. In addition, it was also demonstrated that KL sequences are deleted in the genome of the S1 mouse. Taken together, these results suggest that KL is encoded by the S1 locus and is the ligand of the c-kit receptor, thus providing a molecular basis for the S1 defect.

The amino acid sequence predicted from the nucleotide sequence of the KL cDNA clone suggests that KL is synthesized as an integral transmembrane protein. The structural features of the primary translation product of KL therefore are akin to those of CSF-1. CSF-1 is synthesized as a transmembrane molecule, which is processed by proteolytic cleavage to form a soluble product that is secreted (91, 44). Presumably, like CSF-1, KL is also synthesized as a cell surface molecule that may be processed to form a soluble protein. The protein purified from conditioned medium of BALB/c 3T3 cells then would represent the soluble form of KL that was released from the cell membrane form by proteolytic cleavage. Although the post-translational processing and expression of the KL protein have not yet been characterized, a cell surface-bound form of KL may mediate the cell-cell interactions proposed for the proliferative and migratory functions of the c-kit/W receptor system. In agreement with the notion of a cell membrane-associated form of KL, a soluble c-kit receptor-alkaline phosphatase fusion protein has been shown to bind to the cell surface of BALB/c 3T3 cells but not to fibroblasts derived from SII/SI mice (14).

A most significant aspect of the identification of the ligand of the c-kit receptor lies in the fact that it will facilitate the investigation of the pleiotropic functions of

-65-

81, 82, 83). It will now be of interest to determine the effect of KL on the self-renewal or the differentiation potential of hematopoietic stem cell populations in vitro, possibly in combination with other hematopoietic growth factors, in order to identify the stage(s) where c-kit/KL functions in stem cells. Another possible function for c-kit might be to facilitate the transition from noncycling to cycling cells (31). The increased radiation sensitivity of SIISI^d and of W/W^v mice might suggest such a role in stem cell dynamics; furthermore, the related PDGF receptor is known to promote entry into the cell cycle.

In gametogenesis the W and Sl mutations affect the proliferation and the survival of primordial germ cells, and their migration from the yolk sac splanchnopleure to the genital ridges during early development. In postnatal gametogenesis c-kit expression has been detected in immature and mature oocytes and in spermatogonia A and B as well as in interstitial tissue (39). In melanogenesis c-kit/KL presumable functions in the proliferation and migration of melanoblast from the neural crest to the periphery in early development as well as in mature melanocytes. The availability of KL may now facilitate in vitro studies of the function of the c-kit receptor in these cell systems.

The microenvironment in which c-kit-expressing cells function is defective in Sl mutant mice and is the presumed site where the c-kit ligand is produced. Because of the extrinsic nature of the mutation, the precise identity of the cell types that produce KL in vivo is not known. In in vitro systems that reproduce the genetic defect of the W and the Sl mutations, however, have shed some light on this question. In the long-term bone marrow culture system, SIISI^d adherent cells are defective but the nonadherent

-67-

5 homozygotes, e.g., in germ cell development, S1 may have a more pronounced effect, and in hematopoiesis S1 may cause a more severe anemia; however, it is not known if these differences are a result of different strain backgrounds or are possibly effects of the S1 deletion on neighboring genes (5).

10 The original W mutation is an example of a c-kit null mutation (36). When heterozygous with the normal allele, WI⁺ mice typically have a ventral spot but no coat dilution and no effects on hematopoiesis and gametogenesis. The weak heterozygous phenotype of WI⁺ mice is in contrast to the phenotype of heterozygous SII⁺ mice, which have moderate macrocytic anemia and a diluted coat pigment in addition to
15 a ventral spot and gonads that are reduced in size. Thus 50% gene dosage of KL is limiting and is not sufficient for normal function of the c-kit receptor, yet 50% dosage of the c-kit receptor does not appear to be limiting in most situations.

20 The c-kit receptor system functions in immature progenitor cell populations as well as in more mature cell types in hematopoiesis, gametogenesis, and melanogenesis. Severe S1 or W mutations may block the development of these cell
25 lineages, and therefore a function for the c-kit receptor in more mature cell populations would not be evident. S1 and W mutations in which c-kit/KL function is only partially impaired often reveal effects in more mature cell populations. Numerous weak S1 alleles are known. Their
30 phenotypes, e.g., in gametogenesis and melanogenesis, will be of great value in the elucidation of the pleiotropic functions of the c-kit receptor system.

-69-

(38).

cdNA Library Screening

5 Poly(A) RNA was prepared by oligo(dT)-cellulose chromatography from total RNA of Balb/c 3T3 fibroblast. A custom made plasmid cdNA library was then prepared by Invitrogen Inc. Essentially, double-stranded cdNA was synthesized by oligo dT and random priming. Non-palindromic
10 BstXI linkers were ligated to blunt-ended cdNA and upon digestion with BstXI the cdNA was subcloned into the expression plasmid pCDNAI (Invitrogen). The ligation reaction mixture then was used to transform E. coli MC1061/P3 by the electroporation method to generate the
15 plasmid library. The initial size of the library was approximately 10^7 independent colonies. For screening of the plasmid library an end-labelled oligonucleotide probe described previously was used (38). Hybridization was done in 6X SSC at 63°C and the final wash of the filters was in
20 2X SSC and 0.2% SDS at 63°C. The inserts of recombinant plasmids were released by digestion with HindIII and XbaI and then subcloned into the phage M13mp18 for sequence analysis.

25 PCR amplification (RT-PCR) and sequence determination

Total RNA from tissues and cell lines was prepared by the guanidium isothiocyanate/CsCl centrifugation method of Chirgwin (10). The RT-PCR amplification was carried out
30 essentially as described previously (38). The following primers were used for RT-PCR:

Primer #1: 5'-GCCCAAGCTTCGGTGCCTTTCCTTATG-3' (nt. 94-107);

-71-

RNAse Protection Assay

5 A riboprobe for RNAse protection assays was prepared by linearizing the KL-1 containing pcDNAI plasmid with SpeI. The antisense riboprobe was then synthesized by using SP6 polymerase according to the Promega Gemini kit. Riboprobe labelled to high specific activity was then hybridized to 10 or 20 μ g of total RNA in the presence of 80% formamide at 45°C overnight. The hybridization mixture was digested with 10 RNAse A and T1 (Boehringer-Mannheim) and treated with proteinase K (48) and the protected labelled RNA fragments were analyzed on a 4% urea/polyacrylamide gel. Autoradiograms of RNAse protection assay were analyzed by densitometry and parts of the films were reconstructed on a 15 PhosphoImage analyzer (Molecular Dynamics) for better resolution.

Transient expression of "KL" cDNAs in COS-1 cells

20 For transient expression of KL cDNAs COS-1 cells were transfected with the DEAE-dextran method described previously (20) with minor modifications. Briefly, COS-1 cells were grown to subconfluence one day before use and were trypsinized and reseeded on 150mm petri dishes at a 25 density of 6×10^6 cells per dish. After 24 hours, the cells had reached about 70% confluence and were transfected with 5 μ g of plasmid DNA in the presence of 10% DEAE-dextran (Sigma) for 6 to 12 hours. Medium containing plasmid DNA was removed and the cells were chemically shocked with 10% 30 DMSO/PBS⁺⁺ for exactly 1 minute. Residual DMSO was removed by washing the cells with PBS⁺⁺ twice. Transfected COS-1 cells were grown in DME plus 10% fetal calf serum, 100 mg/ml L-glutamine, and antibiotics.

-73-

autoradiography.

Determination of biological activity of soluble KL

5 Mast cells were grown from bone marrow of adult WBB6 +/+ mice in RPMI-1640 medium supplemented with 10% fetal calf serum, conditioned medium from WEHI-3B cells, non-essential amino acids, sodium pyruvate and 2-mercaptoethanol (RPMI-Complete) as described previously (37). Non-adherent cells
10 were harvested by centrifugation and refed weekly and maintained at a cell density of $<7 \times 10^5$ cells/ml. The mast cell content of cultures was determined weekly by staining cytospin preparations with 1% toluidine blue in methanol. After 4 weeks, cultures routinely contained >95% mast cells
15 and were used for proliferation assay. Supernatants from transfected COS-1 cells were collected from 48 to 72 hours after transfection. The biological activity of soluble KL in the supernatants was assessed by culturing BMNCs with different dilutions of COS-1 cell supernatants in the
20 absence of IL-3. BMNCs were washed three times with complete RPMI and grown in 0.2% IL-3. The following day, cells were harvested and suspended in complete RPMI (minus IL-3) and 10^4 BMNCs in 100 μ l/well were seeded in a 96-well plate. Equal volume of diluted supernatant was added to
25 each well and cultures were incubated for 24 hours at 37°C, 2.5 μ Ci of [3 H]-thymidine/well was then added and incubation was continued for another 6 hours. Cells were harvested on glass fiber filters (GF/C Whatman) and thymidine incorporation was determined in a scintillation counter.
30 Assays were performed in triplicate and the mean value is shown. Standard deviations of measurements typically did not exceed 10% of the mean values.

-75-

analysis of the RT-PCT reaction products shown in Figure 18 indicates a single fragment of approximately 870 bp in the samples from Balb3T3 cells and brain, whereas in the samples from spleen, testis and lung two fragments were seen, approximately 870 and 750 bp in size. For further analysis the two PCR reaction products were subcloned into the mammalian expression vector pCDM8. DNA sequence analysis first indicated that the larger PCR product corresponds to the known KL cDNA sequence, subsequently referred to as KL-1. In the smaller PCR product, however, a segment of 84 nucleotides of the KL coding sequences was lacking, generating an inframe deletion. The deletion endpoints corresponded to exon boundaries in the rat and the human KL genes and it is quite likely that these boundaries are also conserved in the mouse gene (27). Therefore, the smaller PCR product appeared to correspond to an alternatively spliced KL RNA transcript, designated KL-2. The exon missing in KL-2 precedes the transmembrane domain; it contains one of the four N-linked glycosylation sites and includes the known C-terminus (Ala-166 and Ala-167) of the soluble form of KL (58). KL-2 therefore is predicted to encode a truncated version of KL-1 which is presumably synthesized as a transmembrane protein (Figures 17 and 19).

KL-2 Is Expressed In A Tissue Specific Manner

The alternatively spliced transcript KL-2 had been detected in spleen, testis and lung RNA, but not in fibroblasts and brain RNA, suggesting that the expression of KL-2 may be controlled in a tissue specific manner. In order to address this question in more detail the steady state levels of KL-1 and KL-2 RNA transcripts in RNA were determined from a wide variety of tissues by using an RNase protection assay.

-77-

or pCDM8 for transient expression in COS-1 cells. To facilitate transient expression of the KL-1 and KL-2 protein products COS-1 cells were transfected with the KL-1 and KL-2 plasmids by using the DEAE-dextran/DMSO protocol as described herein. KL protein synthesis in the COS-1 cells was shown to be maximal between 72 to 96 hours subsequent to the transfection. In order to determine the biosynthetic characteristics of the KL-1 and KL-2 proteins pulse-chase experiments were carried out. 72 hours subsequent to transfection, cultures were labeled with ^{35}S -methionine (0.5mCi/ml) for 30 minutes and then chased with regular medium. The cell lysate and supernatants then were collected at the indicated times and processed for immunoprecipitation with anti-KL antiserum, prepared by immunizing rabbits with purified murine KL, and analysis by SDS-PAGE (12%). In cells transfected with the KL-1 plasmid, at the end of the labelling period, KL specific protein products of 24, 35, 40 and 45 kD are found (Figure 21). These proteins presumably represent the primary translation product and processed KL protein products which are progressively modified by glycosylation. Increasingly longer chase times reveal the 45 kD form as the mature KL protein product and it is quite likely that this protein represents the cell membrane form of KL. In the supernatant beginning at 30 minutes a 28 kD KL protein product is seen which, with increasing time, increases in amount. Two minor products of 38 and 24 kD were also found with increasing time. These results are consistent with the notion that KL-1 is first synthesized as a membrane-bound protein and then released into the medium probably through proteolytic cleavage.

A pulse-chase experiment of COS-1 cells transfected with the KL-2 plasmid is shown in Figure 20. The KL-2 protein

-79-

inducer. These results suggest that the proteolytic cleavage machinery for both KL-1 and KL-2 is activated similarly by PMA. On one hand this may mean that two distinct proteases, specific for KL-1 and KL-2 respectively, are activated by PMA or alternatively, that there is one protease which is activated to a very high level which cleaves both KL-1 and KL-2 but with different rates. The major cleavage site in KL-1 based on the known C-terminal amino acid sequence of rat KL, includes amino acids PPVA A SSL (186-193) and may involve an elastase like enzyme (22,34). The recognition sequence in KL-2, based on the arguments presented above, presumably lies C-terminal of the deleted exon and therefore might include amino acids RKAAGA (202-207) and thus could involve an enzyme with a specificity similar to the KL-1 protease, alternatively, it could be a trypsin-like protease. The effect of the calcium ionophore A23187 on KL cleavage has been determined. Both KL-1 and KL-2 cleavage is accelerated by this reagent indicating that mechanisms that do not involve the activation of protein kinase C can mediate proteolytic cleavage of both KL-1 and KL-2 (Figure 22C).

Biological activity of the released KL protein products

To test the biological activity of the released KL protein products, the supernatants of transfected COS-1 cells were collected 72 hours after transfection and assayed for activity in the mast cell proliferation assay. Bone marrow derived mast cells (BMMC) were incubated for 24 hours with different dilutions of the collected supernatants and assayed for ³H-thymidine incorporation as described previously (Figure 23). Supernatants from KL-1 transfectants produced 3 to 5 times more activity than KL-2 transfectants in agreement with the differential release of

-81-

the wild-type sequence is deleted, instead, a sequence of 67 bp was found to be inserted (Figure 17). The deletion insertion results in a termination codon three amino acids from the 5' deletion endpoint. The predicted amino acid sequence of KL-S1^d cDNA consists of amino acids 1 - 205 of the known KL sequence plus 3 additional amino acids (Figures 17 and 19). The KL-S1^d amino acid sequence includes all four N-linked glycosylation sites and all sequences contained in the soluble form of KL, while the transmembrane and the cytoplasmic domains of wild-type KL-1 are deleted. Consequently, the KL-S1^d protein product is a secreted protein, which displays biological activity.

Biosynthetic Characteristics And Biological Activity Of The KL-S1^d and KL-S Protein Products

For comparison with the KL-S1^d protein product, a truncated version of KL-1 was made, designated KL-S, in which a termination codon was inserted at amino acid position 191 which is the presumed C-terminus of the soluble KL protein. COS-1 cells were transfected with the KL-S1^d and the KL-S plasmids and pulse-chase experiments were carried out to determine the biosynthetic characteristics of the two protein products. The KL-S1^d protein product is rapidly processed, presumably by glycosylation and then secreted into the medium, where the major 30 kD species is found after as early as 30 minutes of chase time and then increases in amount thereafter (Figure 24). The biosynthetic characteristics of the KL-S protein products are very similar to those of KL-S1^d (Figure 24). Again, with increasing time increasing amounts of secreted material are detected in the medium, conversely the cell associated KL-S protein products decrease with time.

-83-

KL-2 in COS-1 cells is a process that can be modulated. Fourth, KL-1 and KL-2 are expressed in a tissue-specific manner. Furthermore, the viable S1^d mutation was shown to be the result of a deletion that includes the C-terminus of the KL coding sequence including the transmembrane domain generating a biologically active secreted form of KL. The phenotype of mice carrying the S1^d allele provides further support for the concept for a role for both the secreted and the cell membrane-associated forms of KL in c-kit function.

Because of the close evolutionary relationship of c-kit with CSF-1R it was reasonable to predict a relationship between the corresponding growth factors, KL and CSF-1, in regards to both structural and topological aspects. Alternatively spliced forms of CSF-1 mRNAs are known to encode protein products which differ in sequences N-terminal of the transmembrane domain, a spacer segment of 298 amino acids located in between the ligand portion and the transmembrane domain of the protein (43). In addition, alternatively spliced CSF-1 RNA transcripts differ in their 3' untranslated regions (21). Analysis of KL RNA transcripts in several tissues identified an alternatively spliced KL RNA in which, similar to the situation in CSF-1, the spacer between the presumed ligand portion and the transmembrane domain is deleted. Interestingly, the expression of this alternatively spliced RNA product is controlled in a tissue specific manner. A recent comparative analysis of the ligand portions of KL and CSF-1 indicates structural homology between the two proteins based on limited amino acid homology and the comparison of corresponding exons and matching of "exon-encoded secondary structure" (4). Furthermore, the super position of 4 α -helical domains and cysteine residues which form intra-molecular disulfide bonds implies related tertiary structures for the ligand domains

-85-

adhesion molecules (19, 26). On the other hand, the soluble forms of KL are diffusible factors which may reach the target cell and its receptor over a relatively short or longer distances. But the soluble forms of KL might also become associated with, or sequestered in the extracellular matrix, in an analogous fashion to FGF, LIF or int-1, and thus function over a short distance similar to the membrane-associated form (8,33,42). When cell membrane-associated, KL may be able to provide or sustain high concentrations of a localized signal for interaction with receptor-carrying target cells. In turn the soluble form of KL may provide a signal at lower and variable concentrations. c-kit is thought to facilitate cell proliferation, cell migration, cell survival and post-mitotic functions in various cell systems. By analogy with the CSF-1 receptor system, the cell survival function and cell migration might require lower concentrations of the factor than the cell proliferation function (55). The cell membrane-associated and the soluble forms of KL then may serve different aspects of c-kit function. Both the CSF-1 receptor and c-kit can be down-regulated by protein kinase C mediated proteolytic release of the respective extracellular domains (13). The functional significance of this process is not known but it has been hypothesized that the released extracellular domain of these receptors may neutralize CSF-1 and KL, respectively, in order to modulate these signals. In some ways proteolytic cleavage of KL results in a down modulation of c-kit function and the processes, therefore, may be considered as complementary or analogous. In summary, the synthesis of variant cell membrane-associated KL molecules and their proteolytic cleavage to generate soluble forms of KL provide means to control and modulate c-kit function in various cell types during development and in the adult animal.

-87-

5 cells, on one hand, may indicate that the amount of soluble KL-S1^d protein which is released by these cells is not sufficient to facilitate proliferation; on the other hand, these results may suggest that there is a critical role for the cell membrane associated form of KL in this process.

-89-

5299.

- 5 11. Copeland, N.G., Gilbert, D.J., Cho, B.C.,
Donovan, P.J., Jenkins, N.A., Cosman, D.,
Anderson, D., Lyman, S.D. and Williams, D.E.
(1990) Cell 63, 175-183.
- 10 12. Dexter, T.M. and Moore, M.A.S. (1977) Nature
269, 412-414.
13. Downing, J.R., Roussel, M.F. and Sherr, C.J.
(1989) Mol. Cell. Biol. 9, 2890.
- 15 14. Flanagan, J.G. and Leder, P. (1990). Cell 63,
185-194.
15. Flanagan, J.G., Chan, D. and Leder, P. (1991)
Cell 64, 1125-1135.
- 20 16. Fujita, J., Onoue, H., Ebi, Y., Nakayama, H.,
Kanakura, Y. and Kitamura, Y. (1989) Proc.
Natl. Acad. Sci. U.S.A. 86, 2888-2891.
- 25 17. Geissler, E.N., Ryan, M.A. and Housman, D.E.
(1988) Cell 55, 185-192.
18. Gluzman, Y. (1981) Cell 23, 175-182.
19. Gordon, M.Y. (1991) Cancer Cells 3, 127-133.
- 30 20. Kriegler, M. (1990) Gene transfer and expression:
A laboratory manual. (New York; Stockton Press)
21. Ladner, M.B., Martin, G.A., Noble, J.A.,

-91-

30. McCulloch, E.A., Siminovitch, L., Till, J.E., Russel, E.S., and Bernstein, S.E. (1965) *Blood* 26, 399-410.
- 5 31. McCulloch, E.A. (1970) In Regulation of hematopoiesis, A.S. Gordon, ed. (New York: Appleton), pp.649-675.
- 10 32. Mintz, B. and Russell, E.S. (1957) *J. Exp. Zool.* 134, 207-237.
33. Morrison-Graham, K. and Weston, J.A. (1989) *Trends Genet.* 5, 116-121.
- 15 34. Naughton, M.A. and Sanger, F. (1961) *Biochem. J.* 78, 156-162.
- 20 35. Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A. and Besmer, P. (1989) *Genes & Dev.* 3, 816-826.
- 25 36. Nocka, K., Tan, J., Chiu, E., Chu, T.Y., Ray P., Traktman, P. and Besmer, P. (1990a) *EMBO J.* 9, 1805-1813.
37. Nocka, K., Buck, J., Levi, E. and Besmer, P. (1990b) *EMBO J.* 9, 3287-3294.
- 30 38. Nocka, K., Huang, E., Beier, D.R., Chu, T.Y., Buck, J., Lahm, H.W., Wellner, D., Leder, P. and Besmer, P. (1990). *Cell* 63, 225-233.
39. Orr-Urtreger, A., Avivi, A., Zimmer, Y., Givol, D., Yarden Y. and Lonai, P. (1990) *Development*

-93-

Proc. Natl. Acad. Sci. USA 74, 5463-5467.

50. Sarvella, P.A. and Russel, L.B. (1956) J. Hered. 47, 123-128.
51. Silver, W.K. (1979) White-spotting, patch and rump-white. In the Coat Colors of Mice: A model for Gene Action and Interaction (New York: Springer-Verlag), pp. 206-241.
52. Stevens, L.C. (1979) Inbred Strains of mice. 11, 39.
53. Tan, J.C. Nocka, K., Ray, P., Traktman, P. and Besmer, P. (1990) Science 247, 209-212.
54. Todaro, G.J. and Green, H. (1963) J. Cell Biol. 17, 299-313.
55. Tushiniski, R.J., Oliver, I.T., Guilbert, L.J., Tynan, P.W., Warner, J.R. and Stanley, E.R. (1982) Cell 28, 71-81.
56. Williams, D.E., Eisenman, J., Baird, A., Rauch, C., Ness, K.V., March, C.J., Park, L.S., Martin, U., Mochizuki, D.Y., Bosell, H.S., Burgess, G.S., Cosman, D. and Stewart, D.L. (1990) Cell 63, 167-174.
57. Yarden, Y., Kuang, W.J., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T.J., Chen, E., Schlessinger, J., Francke, U. and Ullrich, A. (1987) EMBO J. 6, 3341-3351.

-95-

67. Levi-Schaffer, F., Austen, K.F., Caulfield, J.P.,
Hein, A., Bloes, W.F. and Stevens, R.L. (1985) J.
Immunol. 135, 3454-3462.
- 5 68. Gregory, C.J. and Eaves, A.C. (1978) Blood 51,
527-537.
69. Iscove, N.N. (1978b). In Polastic Anemia, S.
Hibino, S. Takaku and N.T. Shahidi, eds. (Tokyo:
10 University of Tokyo Press), pp. 31-36.
70. Das, S.K. and Stanley, E.R. (198) J. Biol.
Chem. 257, 13679.
- 15 71. Gough, N.M. and Williams, L.R. (1989) Cancer
Cells 1, 77-80.
72. Kitamura, Y., Go, S., and Hatnaka, K. (1978).
Blood 52, 447-452.
- 20 73. Kitamura, Y., and Fujita, J. (1989). Blood 53,
492-497.
74. Nakahata, T., Kobayashi, T., Ishiguro, A., Tsuji,
25 K., Naganuma, K., Ando, O., Yagi, Y., Tadokoro,
K. and Akabane, T. (1986) Nature 324, 65-67.
75. Tsuji, K., Natahata, T., Takagi, M., Kobayashi,
30 T., Ishiguro, A., Kikuchi, T., Naganuma, K.,
Koiki, K., Miyajima, A., Arai, K., Akabane,
T. (1990a) J. Immunol. 144, 678-684.
76. Tsuji, K., Nakahata, T., Takagi, M., Kobayashi,

-97-

142, 2405-2417.

- 5 85. Schmidt, E.V., Paterngale, P.K., Weir, L. and Leder, P. (1988). Proc. Natl, Acad. Sci. USA 85, 6047-6051.
- 10 86. Lehrach, H., Diamond, D., Wozney, J.M., and Boedite, H. (1977). RNA molecular weight determinations by gel electrophoresis under denaturing conditions-a critical reexamination. Biochemistry 16, 4743.
- 15 87. Feinberg, A.P., and Vogeistein, B. (1963). Anal. Biochem. 132, 6-13.
88. Stanley, E.R., and Guilbert, L.J. (1961). J. Immunol. Meth. 42, 263-264.
- 20 89. Scherr, C.J., Rettenmier, C.W., Sacca, R., Roussel, M.F., Look, A.T. and Stanley, E.R. (1965). Cell 41, 666-676.
- 25 90. Chui, D.K., Liato, S.K., and Walker, K. (1978). Blood 51, 539-547.
91. Avner, P., Amar. L., Dandolo, L., and Guenet, J.L. (1968). Trends Gene, 4, 18-23.
- 30 92. Yung, et al. (1981) J. Immunol. 127, 794-799.
93. Stevens, R.L. and Austen, K.F. (1989), Immunol. Today 10, 381-386.
94. Schrader, J.W. (1981) J. Immunol. 126, 452-460.

-99-

What is claimed is:

- 5 1. A purified mammalian protein corresponding to a c-kit ligand which comprises a homodimer of two polypeptides, each polypeptide having a molecular weight of about 30 kilodaltons and an isoelectric point of 3.8.
- 10 2. A purified mammalian protein of claim 1, wherein the mammalian protein is a murine protein.
3. A purified mammalian protein of claim 1, wherein the mammalian protein is a human protein.
- 15 4. A purified mammalian protein corresponding to a c-kit ligand which comprises a homodimer of two polypeptides, each polypeptide having a molecular weight of about 30 kilodaltons, an isoelectric point of 3.8, and wherein the two polypeptides
20 are linked by a disulfide bond.
- 25 5. A pharmaceutical composition comprising the purified mammalian protein of claims 1 or 4 and a pharmaceutically acceptable carrier.
- 30 6. A pharmaceutical composition for the treatment of leucopenia in a mammal, which comprises an effective amount of the pharmaceutical composition of claim 5 and an effective amount of a factor selected from the group consisting of G-CSF, GM-CSF and IL-3, effective to treat leucopenia in a mammal.
7. A pharmaceutical composition for the treatment of

-101-

symptoms of defective lung development which comprises an effective amount of the composition of claim 5, effective to treat infants exhibiting symptoms of defective lung development.

5

13. A composition for the prevention of hair loss in a subject which comprises an effective amount of the pharmaceutical composition of claim 5, effective to prevent hair loss in the subject.

10

14. A composition for inhibiting the loss of pigment in a subject's hair, which comprises an effective amount of the pharmaceutical composition of claim 5, effective to prevent the loss of pigment in the subject's hair.

15

15. An isolated nucleic acid molecule which encodes an amino acid sequence corresponding to a c-kit ligand (KL).

20

16. An isolated nucleic acid molecule of claim 15, wherein the c-kit ligand (KL) is a human c-kit ligand (KL).

25

17. An isolated nucleic acid molecule of claim 15, wherein the c-kit ligand (KL) is a murine c-kit ligand (KL).

30

18. An isolated nucleic acid molecule of claim 15, wherein the nucleic acid molecule is a DNA molecule.

19. The DNA molecule of claim 18, wherein the DNA molecule is a cDNA molecule.

-103-

- 5
30. A vector of claim 28 which comprises a virus.
31. A host vector system for the production of an amino acid sequence which is the c-kit ligand which comprises the plasmid of claim 29 in a suitable host.
- 10
32. A host vector system of claim 31, wherein the suitable host is a eucaryotic cell.
33. A host vector system of claim 32, wherein the eucaryotic cell is a mammalian cell.
- 15
34. A host vector system of claim 32, wherein the eucaryotic cell is an insect cell.
35. A host vector system of claim 32, wherein the eucaryotic cell is a yeast cell.
- 20
36. A host vector system of claim 31, wherein the suitable host is a procaryotic cell.
- 25
37. A c-kit ligand (KL) polypeptide wherein the c-kit ligand (KL) polypeptide comprises a fragment of the protein of claim 1.
- 30
38. A mutated c-kit ligand (KL) polypeptide wherein the biological activity mediated by the binding of the ligand to the receptor is destroyed.
39. A substance capable of specifically forming a complex with the c-kit ligand (KL) polypeptide of claim 1 or 37.

-105-

pharmaceutically acceptable carrier.

- 5 49. A method of modifying a biological function associated with c-kit cellular activity which comprises contacting a cell, whose function is to be modified, with an effective amount of the pharmaceutical composition of claim 5, effective to modify the biological function of the cell.
- 10 50. The method of claim 49, wherein the biological function is the propagation of a cell that expresses c-kit.
- 15 51. The method of claim 50, wherein the cell which expresses c-kit is a hematopoietic cell.
52. The method of claim 49, wherein the biological function is in vitro fertilization.
- 20 53. A method of modifying a biological function associated with c-kit cellular activity in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 5, effective to modify the biological function associated with c-kit function.
- 25 54. A method of stimulating the proliferation of mast cells in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 5, effective to stimulate the proliferation of the mast cells in the patient.
- 30

-107-

- 5 61. A method of treating allergies in a patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 5, effective to treat the allergy.
- 10 62. A method of treating melanoma in a patient, which comprises administering to the patient an effective amount of the composition of claim 5, effective to treat the melanoma.
- 15 63. A method for the treatment of leucopenia in a patient which comprises administering an effective amount of the composition of claim 6.
- 20 64. A method for the treatment of anemia in a patient which comprises administering an effective amount of the composition of claim 7.
- 25 65. A method for enhancing engraftment of bone marrow during transplantation in a patient which comprises administering an effective amount of the composition of claim 8.
- 30 66. A method of enhancing bone marrow recovery in treatment of radiation, chemical, or chemotherapeutic induced bone marrow aplasia or myelosuppression which comprises treating patients with therapeutic effective doses of the composition of claim 9.
67. A method of treating acquired immune deficiency in a patient which comprises administering to the patient a therapeutically effective amount of the

-109-

5

73. A method of treating infants exhibiting symptoms of defective lung development which comprises administering to the infant a therapeutically effective amount of the polypeptide of claim 12.

10

74. A method of preventing the loss of hair in a patient which comprises administering to the patient an effective amount of the composition of claim 13.

15

75. A method of inhibiting the loss of pigment in a subject's hair which comprises administering to the subject an effective amount of the composition of claim 14.

20

76. A method for measuring the biological activity of a c-kit (KL) polypeptide which comprises:

25

a) incubating normal bone-marrow mast cells with a sample of the c-kit ligand (KL) polypeptide under suitable conditions such that the proliferation of the normal bone-marrow mast cells are induced;

30

b) incubating doubly mutant bone-marrow mast cells with a sample of the c-kit ligand (KL) polypeptide under suitable conditions;

c) incubating a. and b. with ^3H -thymidine;

d) determining the amount of thymidine incorporated into the DNA of the normal bone-marrow mast cells and the doubly mutant bone-marrow mast cells; and

1/35

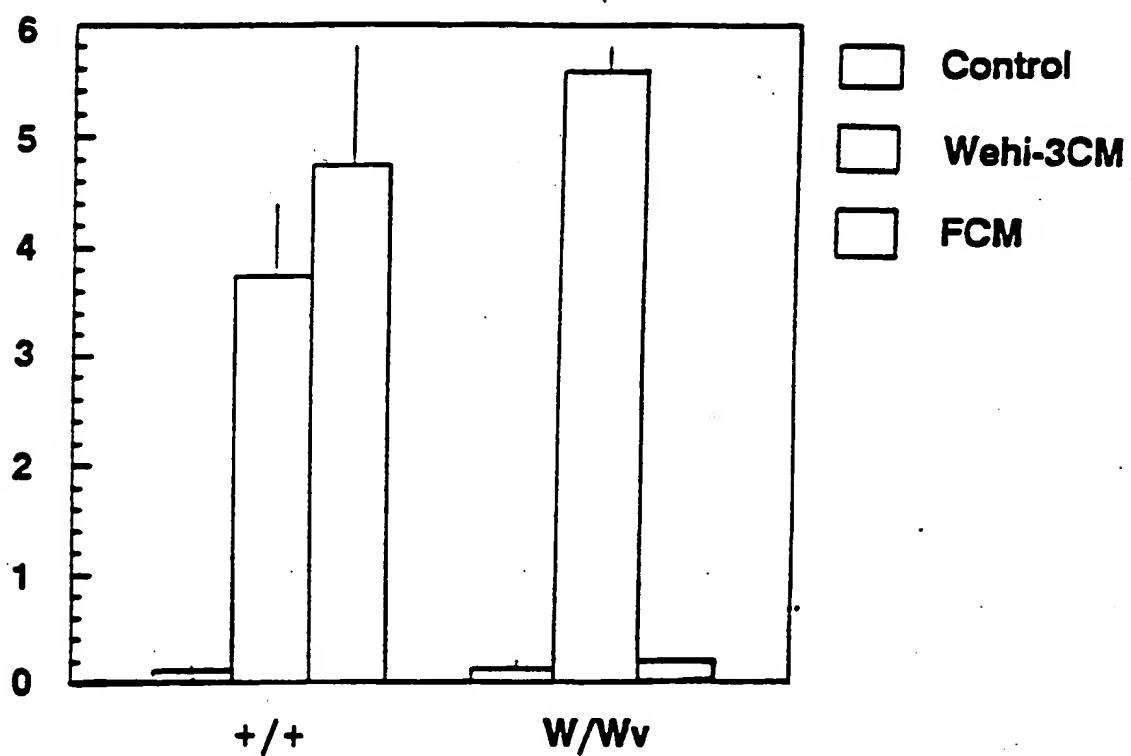
CPM X 10⁻³

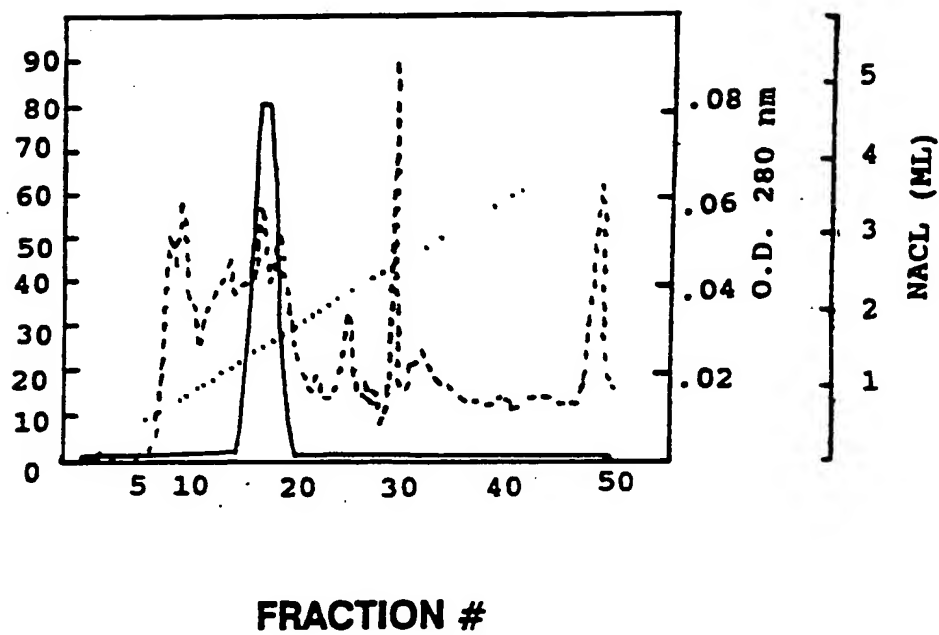
FIGURE 1

SUBSTITUTE SHEET

3/35

FIGURE 2 B

DEAU-5PW



5/35

FIGURE 2 D

ANALYTICAL C 18

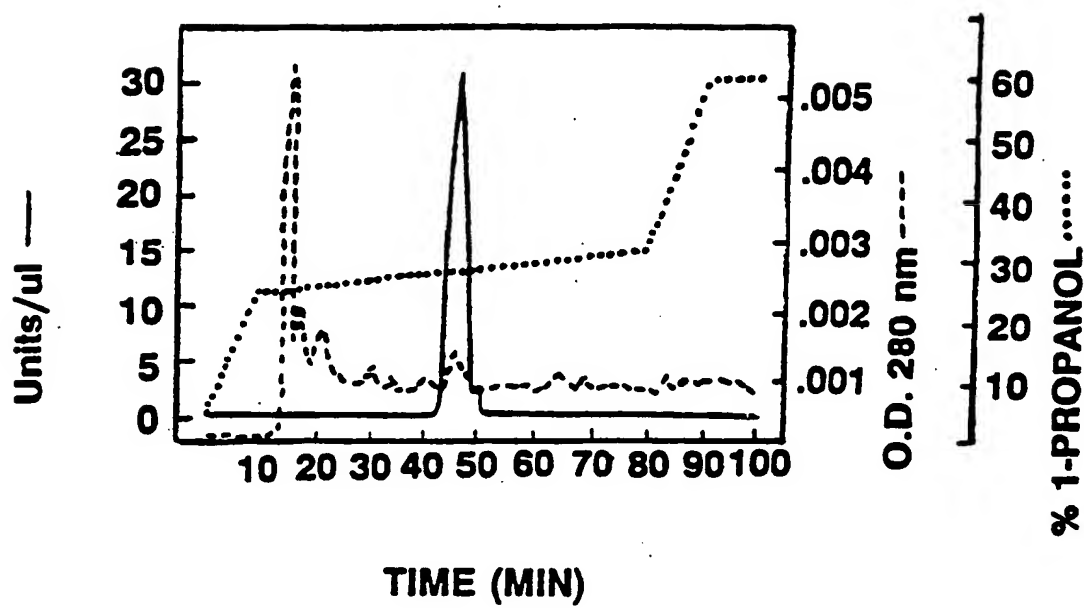
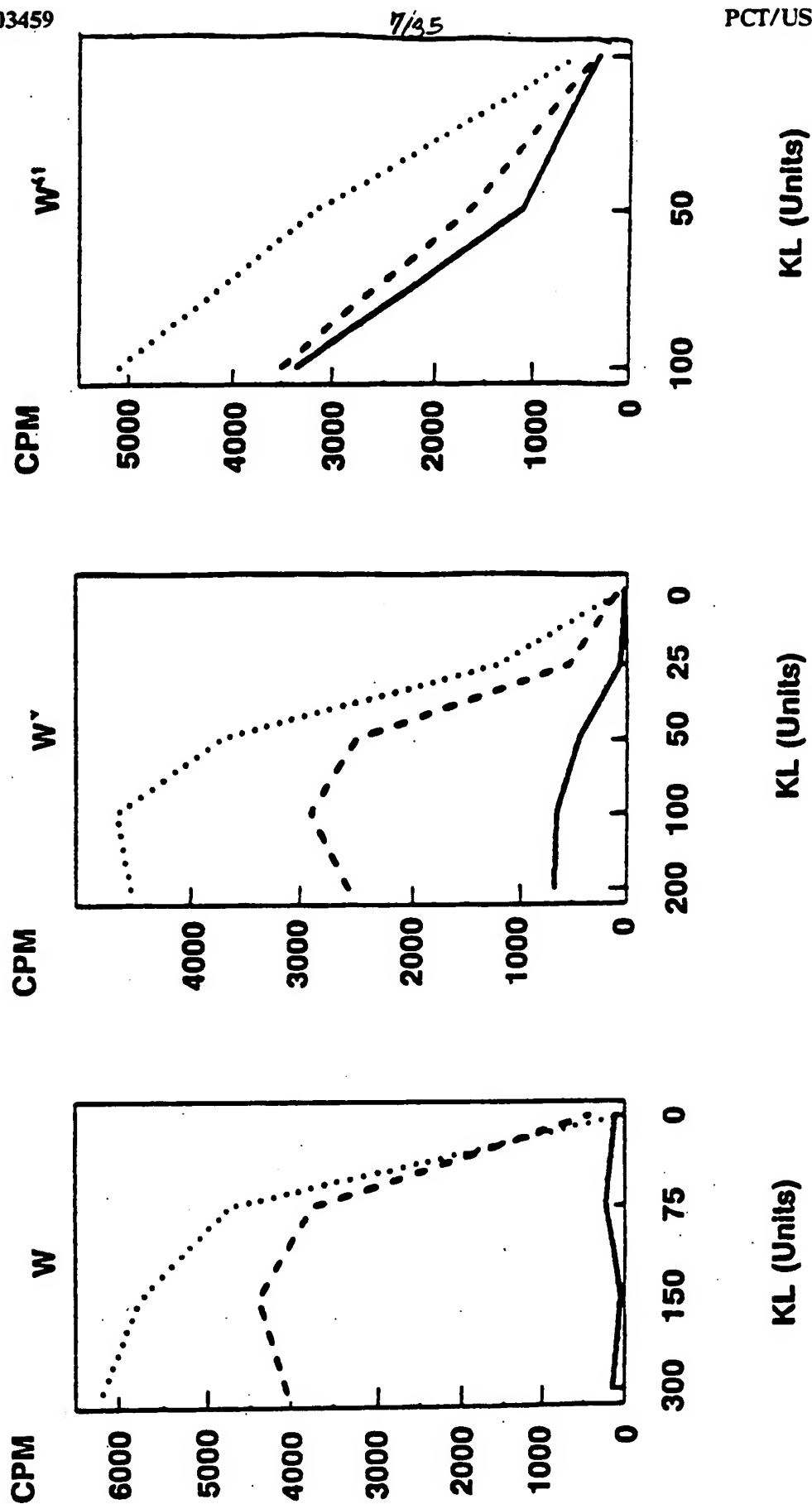


FIGURE 4



9/35

C-KIT SURFACE EXPRESSION

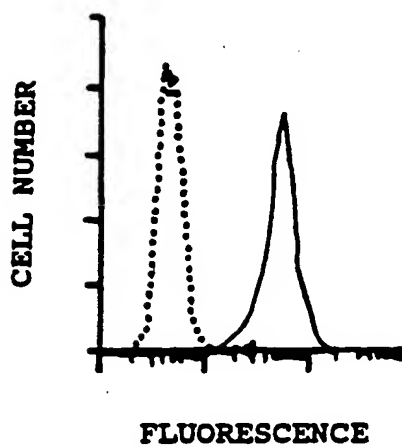
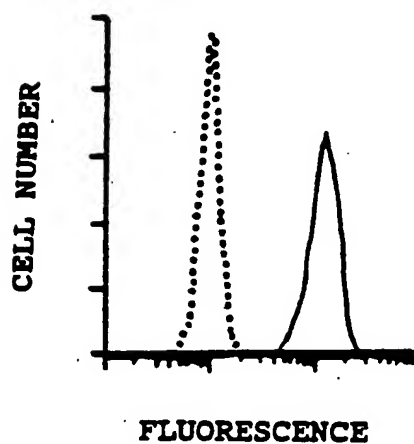
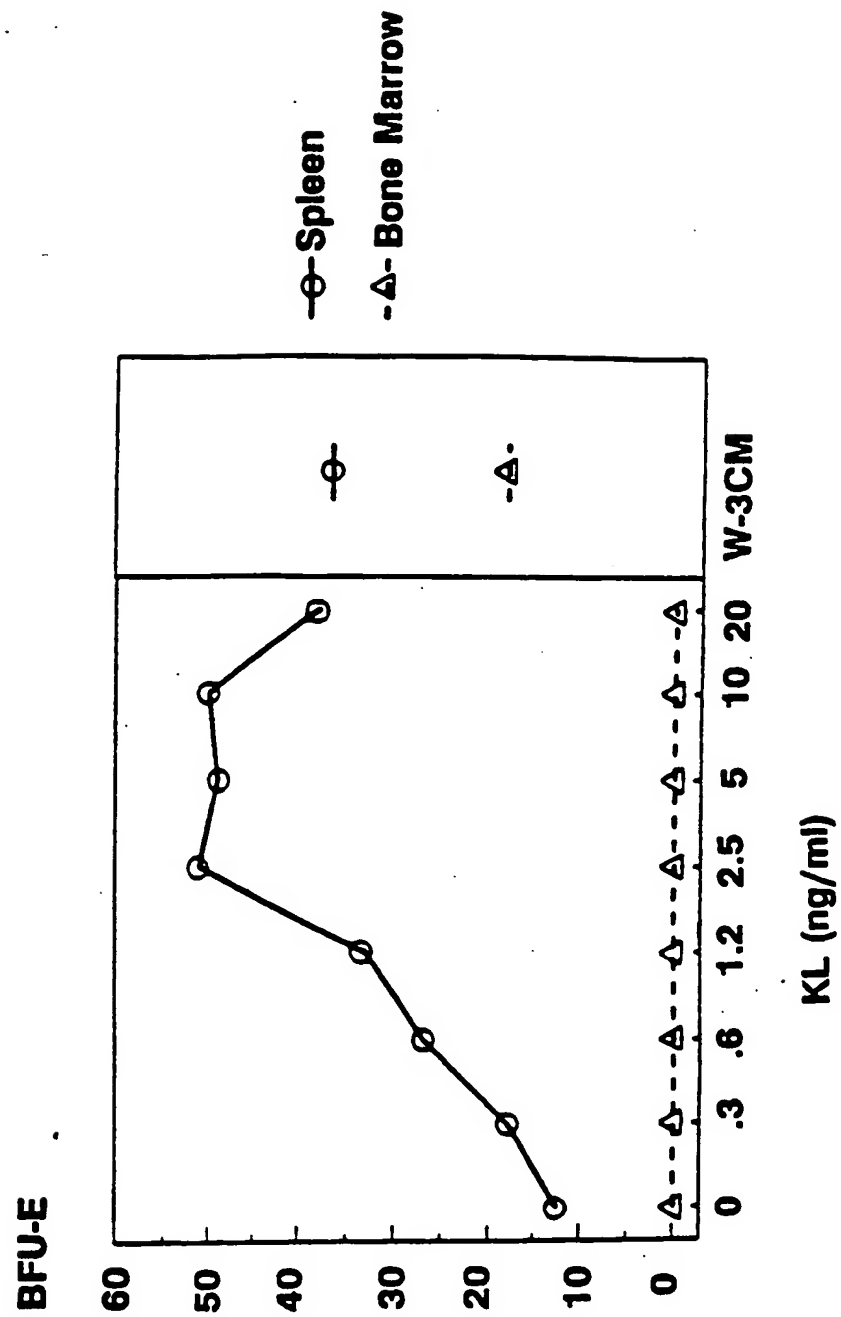


FIGURE 5 B

11/35

FIGURE 6



10 20 30
 KEIXGNPVT D N V K D I T K L V A N L P N O Y N I T L N Y V A G N XVL

5' C G T C 3'
 aagcttgataatgtataaagacattacaaaactggggcnaatcttccnaatgactatataatgataaaccctcaattacgtggccggaatgggatcc

5' cgccaagccttgataatgtataaagatattac 3'
 C C G G C C
 T C A
 3' TTAATACAGGGCCGTACcctagggcc 5'
 G G T T T
 C C C
 A A A

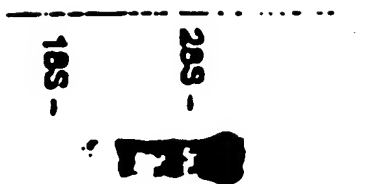
FIGURE 8

15/35



FIGURE 10

17/35



190

FIGURE 12

19/35

FIGURE 14

Figure 14 A

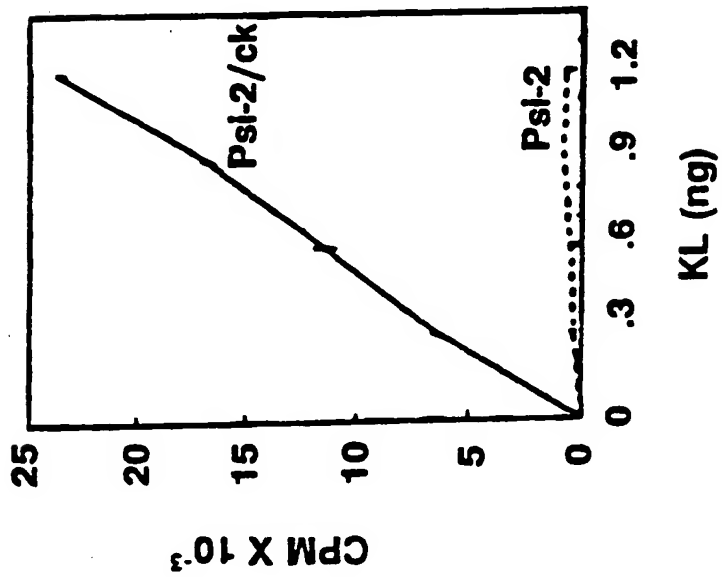
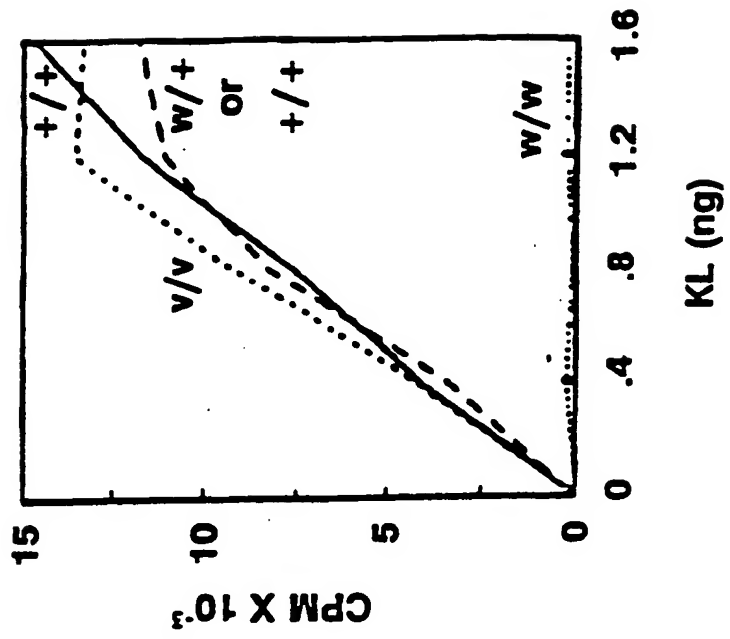


Figure 14B



21/35

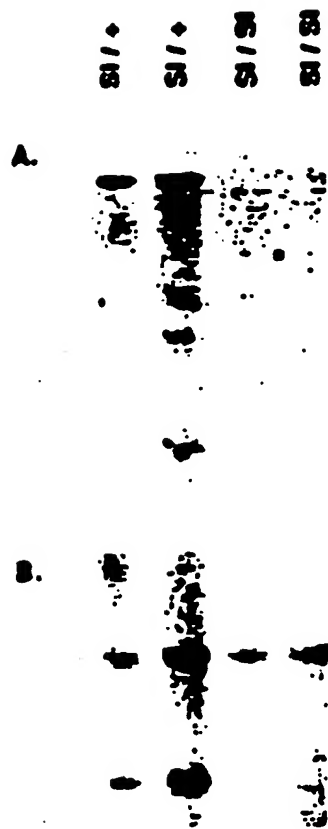


FIGURE 16

23/55

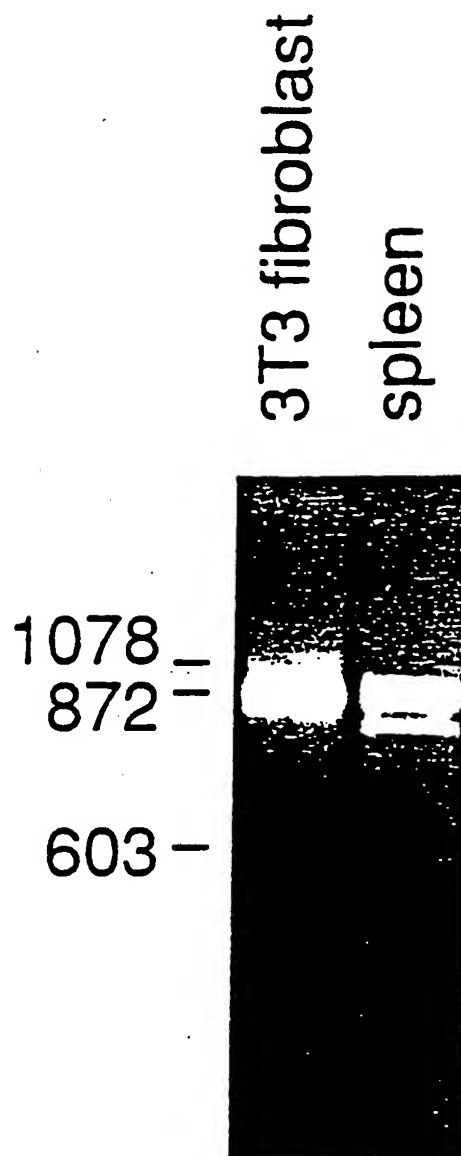


FIGURE 18A

25/35

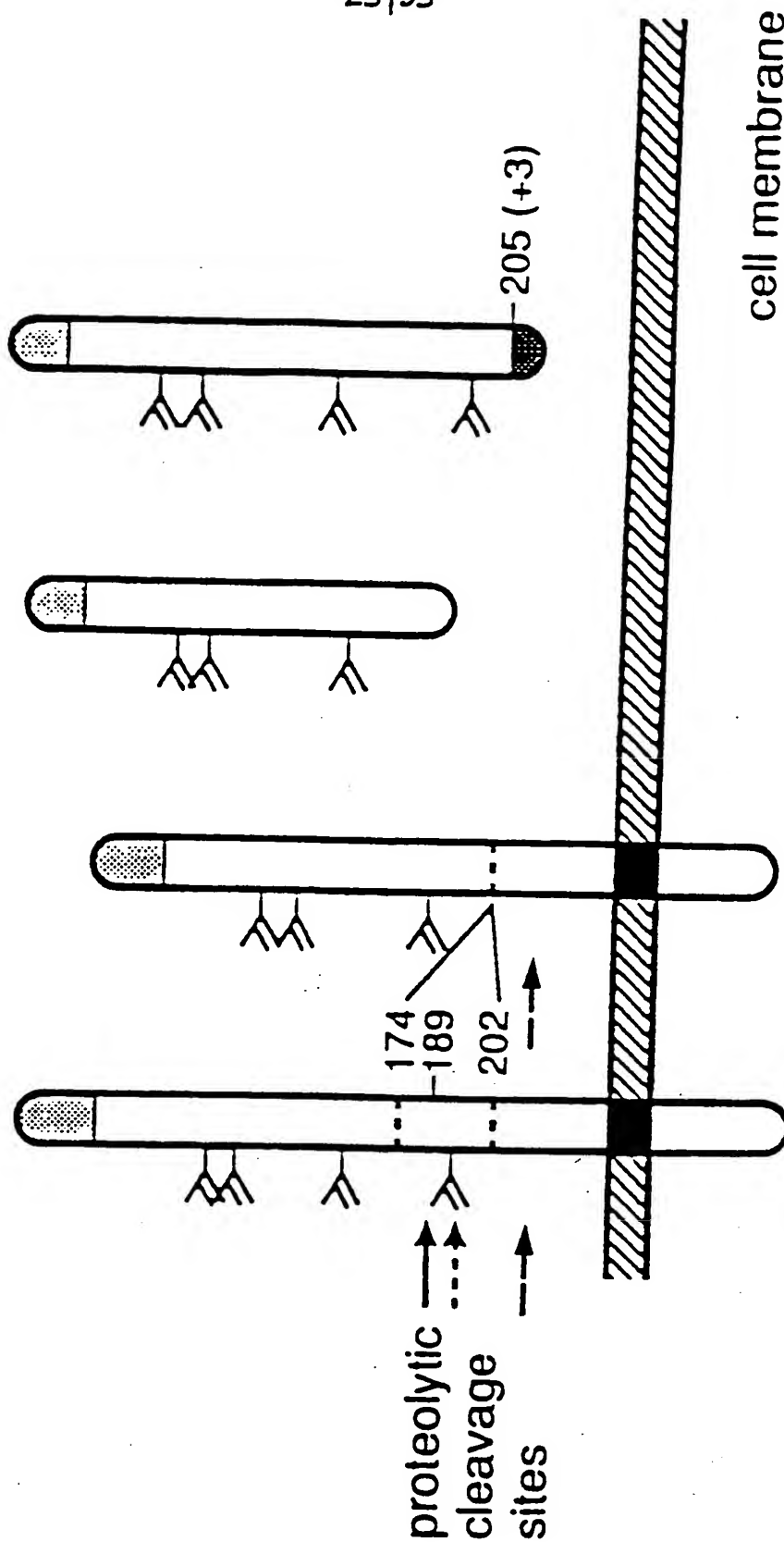


FIGURE 19

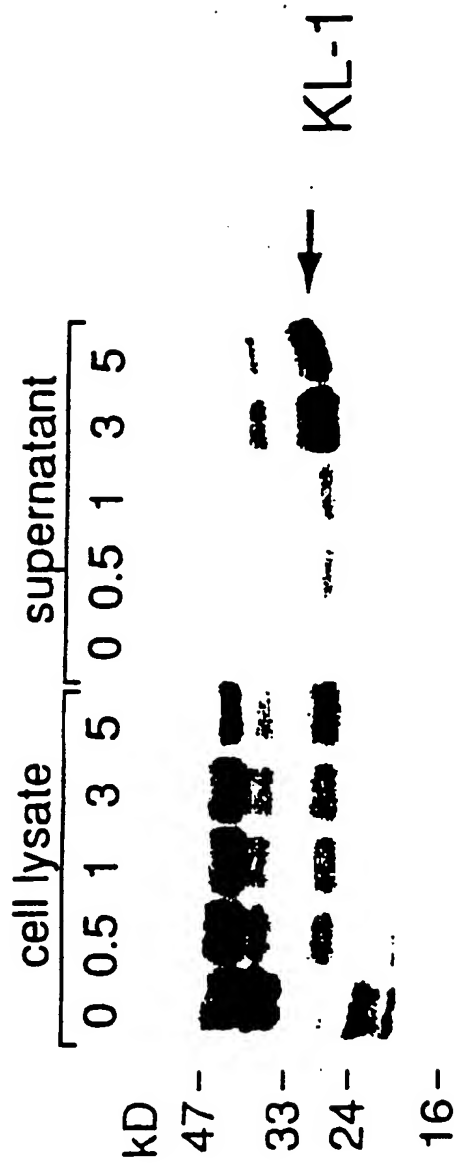


FIGURE 21A

29/35

COS-1

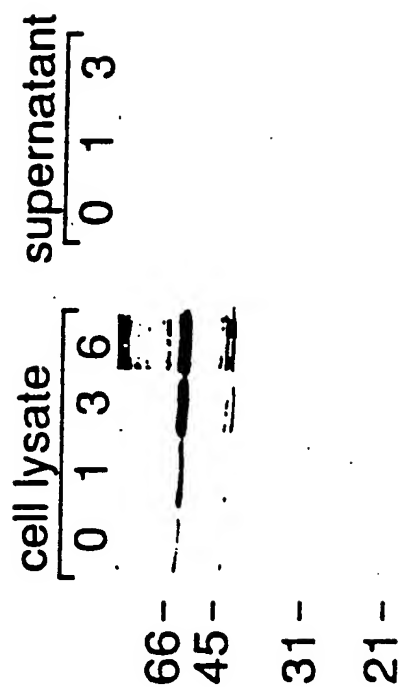


FIGURE 21C

31/35

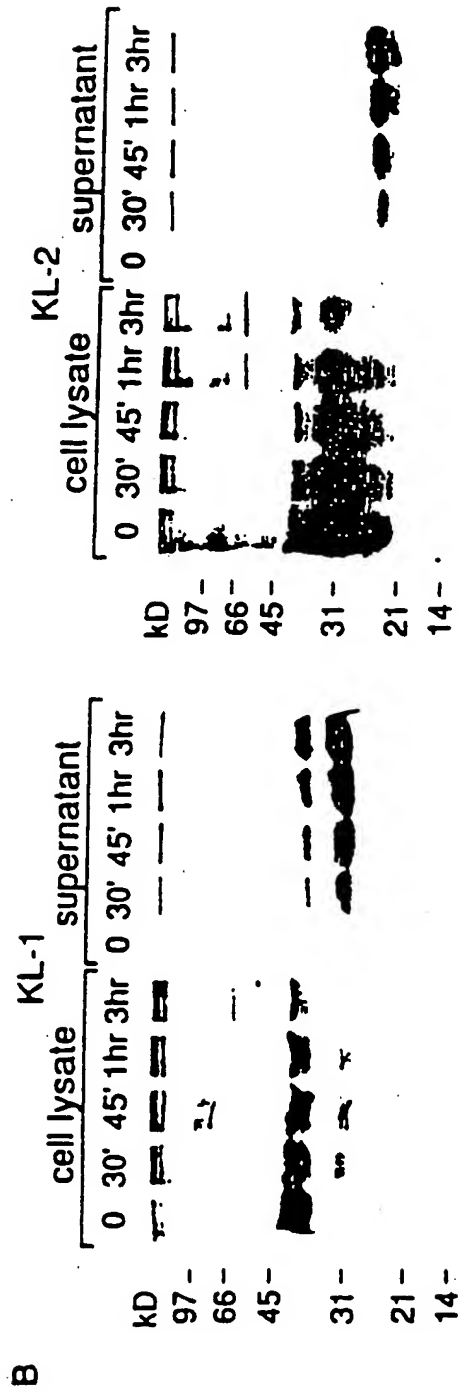


FIGURE 22B

33/35

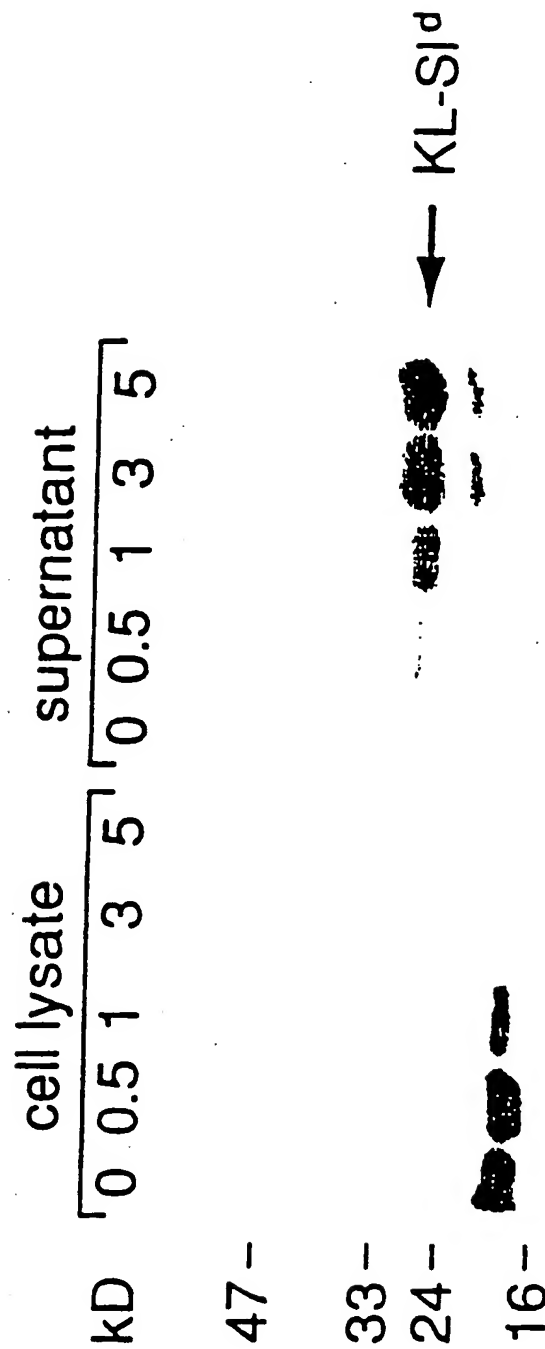


FIGURE 23A

35/35

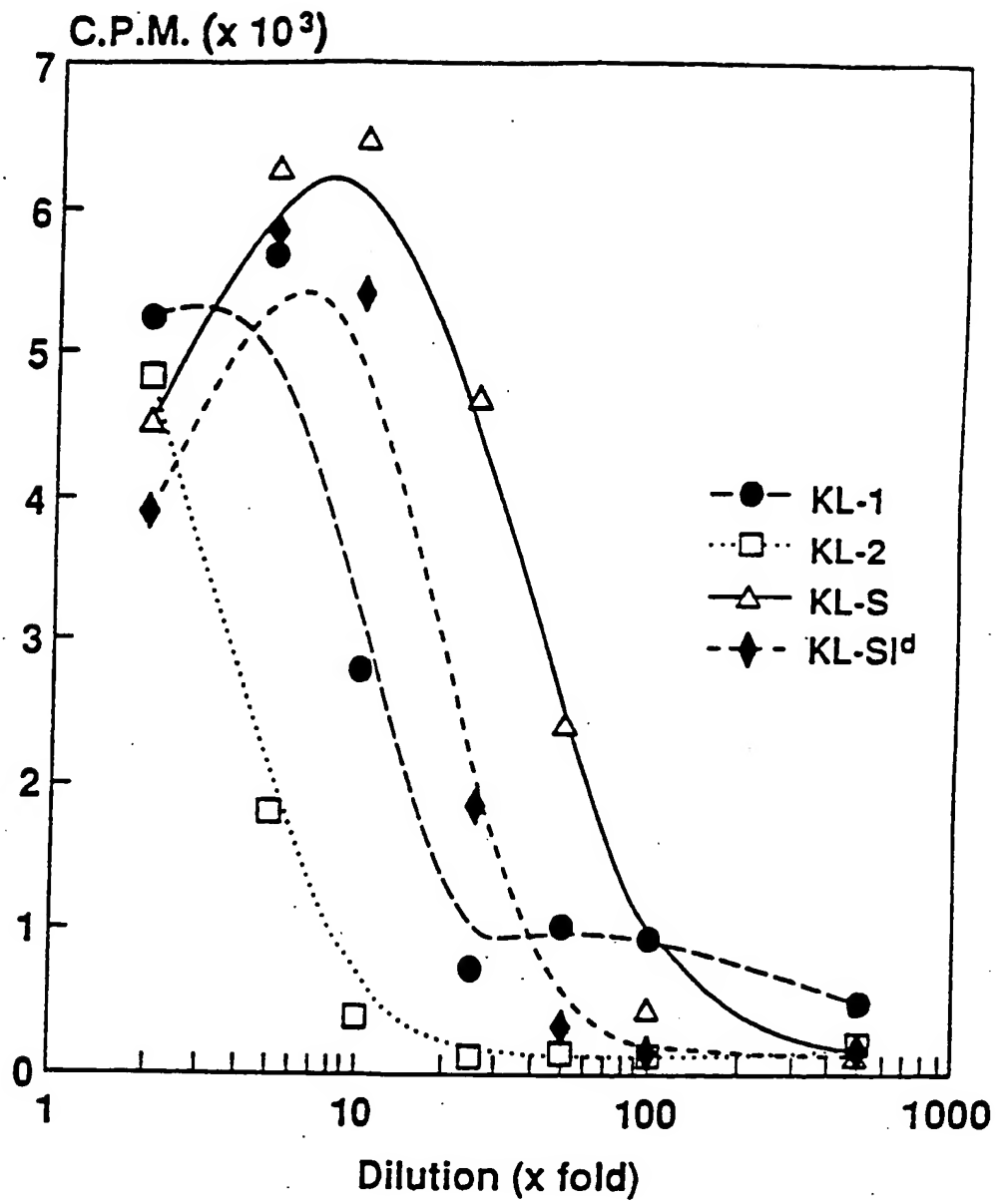


FIGURE 24

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹², specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

See attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-5, 11-37, 46-47, 49-53
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☒ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.